COLD-ADAPTED EQUINE INFLUENZA VIRUSES

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Patent Application Serial No. 09/133,921, filed August 13, 1998; and PCT/US99/18583, filed August 12, 1999; each entitled COLD-ADAPTED EQUINE INFLUENZA VIRUSES. The patent applications referred to in this section are incorporated by reference herein in their entirety.

FIELD OF THE INVENTION

The present invention relates to experimentally-generated cold-adapted equine influenza viruses, and particularly to cold-adapted equine influenza viruses having additional phenotypes, such as attenuation, dominant interference, or temperature sensitivity. The invention also includes reassortant influenza A viruses which contain at least one genome segment from such an equine influenza virus, such that the reassortant virus includes certain phenotypes of the donor equine influenza virus. The invention further includes genetically-engineered equine influenza viruses, produced through reverse genetics, which comprise certain identifying phenotypes of a cold-adapted equine influenza virus of the present invention. The present invention also relates to the use of these viruses in therapeutic compositions to protect animals from diseases caused by influenza viruses.

BACKGROUND OF THE INVENTION

Equine influenza virus has been recognized as a major respiratory pathogen in horses since about 1956. Disease symptoms caused by equine influenza virus can be severe, and are often followed by secondary bacterial infections. Two subtypes of equine influenza virus are recognized, namely subtype-1, the prototype being A/Equine/Prague/1/56 (H7N7), and subtype-2, the prototype being A/Equine/Miami/1/63 (H3N8). Presently, the predominant

virus subtype is subtype-2, which has further diverged among Eurasian and North American isolates in recent years.

The currently licensed vaccine for equine influenza is an inactivated (killed) virus vaccine. This vaccine provides minimal, if any, protection for horses, and can produce undesirable side effects, for example, inflammatory reactions at the site of injection. See, e.g., Mumford, 1987, *Equine Infectious Disease IV*, 207-217, and Mumford, et al., 1993, *Vaccine 11*, 1172-1174. Furthermore, current modalities cannot be used in young foals, because they cannot overcome maternal immunity, and can induce tolerance in a younger animal. Based on the severity of disease, there remains a need for safe, effective therapeutic compositions to protect horses against equine influenza disease.

Production of therapeutic compositions comprising cold-adapted human influenza viruses is described, for example, in Maassab, et al., 1960, *Nature* 7,612-614, and Maassab, et al., 1969, *J. Immunol. 102*, 728-732. Furthermore, these researchers noted that cold-adapted human influenza viruses, i.e., viruses that have been adapted to grow at lower than normal temperatures, tend to have a phenotype wherein the virus is temperature sensitive; that is, the virus does not grow well at certain higher, non-permissive temperatures at which the wild-type virus will grow and replicate. Various cold-adapted human influenza A viruses, produced by reassortment with existing cold-adapted human influenza A viruses, have been shown to elicit good immune responses in vaccinated individuals, and certain live attenuated cold-adapted reassortant human influenza A viruses have proven to protect humans against challenge with wild-type virus. See, e.g., Clements, et al., 1986, *J. Clin. Microbiol. 23*, 73-76. In U.S. Patent No. 5,149,531, by Youngner, et al., issued September 22, 1992, the inventors of the present invention further demonstrated that certain reassortant cold-adapted human influenza A viruses also possess a dominant interference phenotype, i.e.,

they inhibit the growth of their corresponding parental wild-type strain, as well as heterologous influenza A viruses.

U.S. Patent No. 4,683,137, by Coggins et al., issued July 28, 1987, and U.S. Patent No. 4,693,893, by Campbell, issued September 15, 1987, disclose attenuated therapeutic compositions produced by reassortment of wild-type equine influenza viruses with attenuated, cold-adapted human influenza A viruses. Although these therapeutic compositions appear to be generally safe and effective in horses, they pose a significant danger of introducing into the environment a virus containing both human and equine influenza genes.

SUMMARY OF THE INVENTION

The present invention provides experimentally-generated cold-adapted equine influenza viruses, reassortant influenza A viruses that comprise at least one genome segment of an equine influenza virus generated by cold-adaptation such that the equine influenza virus genome segment confers at least one identifying phenotype of a cold-adapted equine influenza virus on the reassortant virus, and genetically-engineered equine influenza viruses, produced through reverse genetics, which comprise at least one identifying phenotype of a cold-adapted equine influenza virus. Identifying phenotypes include cold-adaptation, temperature sensitivity, dominant interference, and attenuation. The invention further provides a therapeutic composition to protect an animal against disease caused by an influenza A virus, where the therapeutic composition includes a cold-adapted equine influenza virus a reassortant influenza A virus, or a genetically-engineered equine influenza virus of the present invention. Also provided is a method to protect an animal from diseases caused by an influenza A virus which includes the administration of such a therapeutic composition. Also provided are methods to produce a cold-adapted equine influenza virus,

and methods to produce a reassortant influenza A virus which comprises at least one genome segment of a cold-adapted equine influenza virus, where the equine influenza genome segment confers on the reassortant virus at least one identifying phenotype of the cold-adapted equine influenza virus.

A cold-adapted equine influenza virus is one that replicates in embryonated chicken eggs at a temperature ranging from about 26°C to about 30°C. Preferably, a cold-adapted equine influenza virus, reassortant influenza A virus, or genetically-engineered equine influenza virus of the present invention is attenuated, such that it will not cause disease in a healthy animal.

In one embodiment, a cold-adapted equine influenza virus, reassortant influenza A virus, or genetically-engineered equine influenza virus of the present invention is also temperature sensitive, such that the virus replicates in embryonated chicken eggs at a temperature ranging from about 26°C to about 30°C, forms plaques in tissue culture cells at a permissive temperature of about 34°C, but does not form plaques in tissue culture cells at a non-permissive temperature of about 39°C.

In one embodiment, such a temperature sensitive virus comprises two mutations: a first mutation that inhibits plaque formation at a temperature of about 39°C, that mutation co-segregating with the genome segment that encodes the viral nucleoprotein gene; and a second mutation that inhibits all viral protein synthesis at a temperature of about 39°C.

In another embodiment, a cold-adapted, temperature sensitive equine influenza virus of the present invention replicates in embryonated chicken eggs at a temperature ranging from about 26°C to about 30°C, forms plaques in tissue culture cells at a permissive temperature of about 34°C, but does not form plaques in tissue culture cells or express late viral proteins at a non-permissive temperature of about 37°C.

Typically, a cold-adapted equine influenza virus of the present invention is produced by passaging a wild-type equine influenza virus one or more times, and then selecting viruses that stably grow and replicate at a reduced temperature. A cold-adapted equine influenza virus produced thereby includes, in certain embodiments, a dominant interference phenotype, that is, the virus, when co-infected with a parental equine influenza virus or heterologous wild-type influenza A virus, will inhibit the growth of that virus.

Examples of cold-adapted equine influenza viruses of the present invention include EIV-P821, identified by accession No. ATCC VR-2625; EIV-P824, identified by accession No. ATCC VR-2624; EIV-MSV+5, identified by accession No. ATCC VR-627; and progeny of such viruses.

Therapeutic compositions of the present invention include from about 10^5 $TCID_{50}$ units to about 10^8 $TCID_{50}$ units, and preferably about 2 x 10^6 $TCID_{50}$ units, of a cold-adapted equine influenza virus, reassortant influenza A virus, or genetically-engineered equine influenza virus of the present invention.

The present invention also includes a method to protect an animal from disease caused by an influenza A virus, which includes the step of administering to the animal a therapeutic composition including a cold-adapted equine influenza virus, a reassortant influenza A virus, or a genetically-engineered equine influenza virus of the present invention. Preferred animals to protect include equids, with horses and ponies being particularly preferred.

Yet another embodiment of the present invention is a method to generate a cold-adapted equine influenza virus. The method includes the steps of passaging a wild-type equine influenza virus; and selecting viruses that grow at a reduced temperature. In one embodiment, the method includes repeating the passaging and selection steps one or more

times, while progressively reducing the temperature. Passaging of equine influenza virus preferably takes place in embryonated chicken eggs.

Another embodiment is an method to produce a reassortant influenza A virus through genetic reassortment of the genome segments of a donor cold-adapted equine influenza virus of the present invention with the genome segments of a recipient influenza A virus. Reassortant influenza A viruses of the present invention are produced by a method that includes the steps of: (a) mixing the genome segments of a donor cold-adapted equine influenza virus with the genome segments of a recipient influenza A virus, and (b) selecting viruses which include at least one identifying phenotype of the donor equine influenza virus. Identifying phenotypes include cold-adaptation, temperature sensitivity, dominant interference, and attenuation. Preferably, such reassortant viruses at least include the attenuation phenotype of the donor virus. A typical reassortant virus will have the antigenicity of the recipient virus, that is, it will retain the hemagglutinin (HA) and neuraminidase (NA) phenotypes of the recipient virus.

The present invention further provides methods to propagate cold-adapted equine influenza viruses or reassortant influenza A viruses of the present invention. These methods include propagation in embryonated chicken eggs or in tissue culture cells.

The present invention also describes nucleic acid molecules encoding wild-type and cold-adapted equine influenza proteins M, HA, NS, PB2, PB2-N, PB2-C, PB1, PB1-N, PB1-C, and PA-C. One embodiment of the present invention is an isolated equine nucleic acid molecule having a nucleic acid sequence selected from a group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12,SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:25 SEQ ID NO:44,

SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:59,SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:76, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:82, SEQ ID NO:85, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:96, SEQ ID NO:103, SEQ ID NO:105, SEQ ID NO:106 and SEQ ID NO:108 and a nucleic acid molecule comprising a nucleic acid sequence which is fully complementary to any of such nucleic acid sequences. Another embodiment of the present invention is an isolated equine nucleic acid molecule that encodes a protein comprising an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:14, SEQ ID NO:17, SEQ ID NO:20, SEQ ID NO:24, SEQ ID NO:45, SEQ ID NO:48, SEQ ID NO:51, SEQ ID NO:55, SEQ ID NO:58, SEQ ID NO:63, SEQ ID NO:66, SEQ ID NO:69, SEQ ID NO:77, SEQ ID NO:81, SEQ ID NO:86, SEQ ID NO:89, SEQ ID NO:92, SEQ ID NO:95, SEQ ID NO:104 and SEQ ID NO:107. Another embodiment is an isolated equine influenza protein that comprises an amino acid sequence selected from a group consisting of SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:14, SEQ ID NO:17, SEQ ID NO:20, SEQ ID NO:24, SEQ ID NO:45, SEQ ID NO:48, SEQ ID NO:51, SEQ ID NO:55, SEQ ID NO:58, SEQ ID NO:63, SEQ ID NO:66, SEQ ID NO:69, SEQ ID NO:77, SEQ ID NO:81, SEQ ID NO:86, SEQ ID NO:89, SEQ ID NO:92, SEQ ID NO:95, SEQ ID NO:104 and SEQ ID NO:107. Also included in the present invention is a virus that include any of these nucleic acid molecules or proteins. In one embodiment, such a virus is equine influenza virus or a reassortant virus.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides experimentally-generated cold-adapted equine influenza viruses comprising certain defined phenotypes, which are disclosed herein. It is to be noted that the term "a" or "an" entity, refers to one or more of that entity; for example, "a cold-adapted equine influenza virus" can include one or more cold-adapted equine influenza viruses. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein. It is also to be noted that the terms "comprising," "including," and "having" can be used interchangeably. Furthermore, an item "selected from the group consisting of" refers to one or more of the items in that group, including combinations thereof.

A cold-adapted equine influenza virus of the present invention is a virus that has been generated in the laboratory, and as such, is not a virus as occurs in nature. Since the present invention also includes those viruses having the identifying phenotypes of such a cold-adapted equine influenza virus, an equine influenza virus isolated from a mixture of naturally-occurring viruses, i.e., removed from its natural milieu, but having the claimed phenotypes, is included in the present invention. A cold-adapted equine influenza virus of the present invention does not require any specific level of purity. For example, a cold-adapted equine influenza virus grown in embryonated chicken eggs may be in a mixture with the allantoic fluid (AF), and a cold-adapted equine influenza virus grown in tissue culture cells may be in a mixture with disrupted cells and tissue culture medium.

As used herein, an "equine influenza virus" is an influenza virus that infects and grows in equids, e.g., horses or ponies. As used herein, "growth" of a virus denotes the ability of the virus to reproduce or "replicate" itself in a permissive host cell. As such, the terms, "growth of a virus" and "replication of a virus" are used interchangeably herein.

Growth or replication of a virus in a particular host cell can be demonstrated and measured by standard methods well-known to those skilled in the art of virology. For example, samples containing infectious virus, e.g., as contained in nasopharyngeal secretions from an infected horse, are tested for their ability to cause cytopathic effect (CPE), e.g., virus plaques, in tissue culture cells. Infectious virus may also be detected by inoculation of a sample into the allantoic cavity of embryonated chicken eggs, and then testing the AF of eggs thus inoculated for its ability to agglutinate red blood cells, i.e., cause hemagglutination, due to the presence of the influenza virus hemagglutinin (HA) protein in the AF.

Naturally-occurring, i.e., wild-type, equine influenza viruses replicate well at a temperature from about 34°C to about 39°C. For example, wild-type equine influenza virus replicates in embryonated chicken eggs at a temperature of about 34°C, and replicates in tissue culture cells at a temperature from about 34°C to about 39°C. As used herein, a "cold-adapted" equine influenza virus is an equine influenza virus that has been adapted to grow at a temperature lower than the optimal growth temperature for equine influenza virus. One example of a cold-adapted equine influenza virus of the present invention is a virus that replicates in embryonated chicken eggs at a temperature of about 30°C. A preferred cold-adapted equine influenza virus of the present invention replicates in embryonated chicken eggs at a temperature of about 28°C. Another preferred cold-adapted equine influenza virus of the present invention replicates in embryonated chicken eggs at a temperature of about 26°C. In general, preferred cold-adapted equine influenza viruses of the present invention replicate in embryonated chicken eggs at a temperature ranging from about 26°C to about 30°C, i.e., at a range of temperatures at which a wild-type virus will grow poorly or not at all. It should be noted that the ability of such viruses to replicate within that temperature range does not preclude their ability to also replicate at higher or lower temperatures. For example,

one embodiment is a cold-adapted equine influenza virus that replicates in embryonated chicken eggs at a temperature of about 26°C, but also replicates in tissue culture cells at a temperature of about 34°C. As with wild-type equine influenza viruses, cold-adapted equine influenza viruses of the present invention generally form plaques in tissue culture cells, for example Madin Darby Canine Kidney Cells (MDCK) at a temperature of about 34°C. Examples of suitable and preferred cold-adapted equine influenza viruses of the present invention are disclosed herein.

One embodiment of the present invention is a cold-adapted equine influenza virus that is produced by a method which includes passaging a wild-type equine influenza virus, and then selecting viruses that grow at a reduced temperature. Cold-adapted equine influenza viruses of the present invention can be produced, for example, by sequentially passaging a wild-type equine influenza virus in embryonated chicken eggs at progressively lower temperatures, thereby selecting for certain members of the virus mixture which stably replicate at the reduced temperature. An example of a passaging procedure is disclosed in detail in the Examples section. During the passaging procedure, one or more mutations appear in certain of the single-stranded RNA segments comprising the influenza virus genome, which alter the genotype, i.e., the primary nucleotide sequence of those RNA segments. As used herein, a "mutation" is an alteration of the primary nucleotide sequence of any given RNA segment making up an influenza virus genome. Examples of mutations include substitution of one or more nucleotides, deletion of one or more nucleotides, insertion of one or more nucleotides, or inversion of a stretch of two or more nucleotides. By selecting for those members of the virus mixture that stably replicate at a reduced temperature, a virus with a cold-adaptation phenotype is selected. As used herein, a "phenotype" is an observable or measurable characteristic of a biological entity such as a cell

or a virus, where the observed characteristic is attributable to a specific genetic configuration of that biological entity, i.e., a certain genotype. As such, a cold-adaptation phenotype is the result of one or more mutations in the virus genome. As used herein, the terms "a mutation," "a genome," "a genotype," or "a phenotype"refer to one or more, or at least one mutation, genome, genotype, or phenotype, respectively.

Additional, observable phenotypes in a cold-adapted equine influenza virus may occur, and will generally be the result of one or more additional mutations in the genome of such a virus. For example, a cold-adapted equine influenza virus of the present invention may, in addition, be attenuated, exhibit dominant interference, and/or be temperature sensitive.

In one embodiment, a cold-adapted equine influenza virus of the present invention has a phenotype characterized by attenuation. A cold-adapted equine influenza virus is "attenuated," when administration of the virus to an equine influenza virus-susceptible animal results in reduced or absent clinical signs in that animal, compared to clinical signs observed in animals that are infected with wild-type equine influenza virus. For example, an animal infected with wild-type equine influenza virus will display fever, sneezing, coughing, depression, and nasal discharges. In contrast, an animal administered an attenuated, cold-adapted equine influenza virus of the present invention will display minimal or no, i.e., undetectable, clinical disease signs.

In another embodiment, a cold-adapted equine influenza virus of the present invention comprises a temperature sensitive phenotype. As used herein, a temperature sensitive cold-adapted equine influenza virus replicates at reduced temperatures, but no longer replicates or forms plaques in tissue culture cells at certain higher growth temperatures at which the wild-type virus will replicate and form plaques. While not being

bound by theory, it is believed that replication of equine influenza viruses with a temperature sensitive phenotype is largely restricted to the cool passages of the upper respiratory tract, and does not replicate efficiently in the lower respiratory tract, where the virus is more prone to cause disease symptoms. A temperature at which a temperature sensitive virus will grow is referred to herein as a "permissive" temperature for that temperature sensitive virus, and a higher temperature at which the temperature sensitive virus will not grow, but at which a corresponding wild-type virus will grow, is referred to herein as a "non-permissive" temperature for that temperature sensitive virus. For example, certain temperature sensitive cold-adapted equine influenza viruses of the present invention replicate in embryonated chicken eggs at a temperature at or below about 30°C, preferably at about 28°C or about 26°C, and will form plaques in tissue culture cells at a permissive temperature of about 34°C, but will not form plaques in tissue culture cells at a non-permissive temperature of about 39°C. Other temperature sensitive cold-adapted equine influenza viruses of the present invention replicate in embryonated chicken eggs at a temperature at or below about 30°C, preferably at about 28°C or about 26°C, and will form plaques in tissue culture cells at a permissive temperature of about 34°C, but will not form plaques in tissue culture cells at a non-permissive temperature of about 37°C.

Certain cold-adapted equine influenza viruses of the present invention have a dominant interference phenotype; that is, they dominate an infection when co-infected into cells with another influenza A virus, thereby impairing the growth of that other virus. For example, when a cold-adapted equine influenza virus of the present invention, having a dominant interference phenotype, is co-infected into MDCK cells with the wild-type parental equine influenza virus, A/equine/Kentucky/1/91 (H3N8), growth of the parental virus is impaired. Thus, in an animal that has recently been exposed to, or may be soon exposed to,

a virulent influenza virus, i.e., an influenza virus that causes disease symptoms, administration of a therapeutic composition comprising a cold-adapted equine influenza virus having a dominant interference phenotype into the upper respiratory tract of that animal will impair the growth of the virulent virus, thereby ameliorating or reducing disease in that animal, even in the absence of an immune response to the virulent virus.

Dominant interference of a cold-adapted equine influenza virus having a temperature sensitive phenotype can be measured by standard virological methods. For example, separate monolayers of MDCK cells can be infected with (a) a virulent wild-type influenza A virus, (b) a temperature sensitive, cold-adapted equine influenza virus, and (c) both viruses in a co-infection, with all infections done at multiplicities of infection (MOI) of about 2 plaque forming units (pfu) per cell. After infection, the virus yields from the various infected cells are measured by duplicate plaque assays performed at the permissive temperature for the cold-adapted equine influenza virus and at the non-permissive temperature of that virus. A cold adapted equine influenza virus having a temperature sensitive phenotype is unable to form plaques at its non-permissive temperature, while the wild-type virus is able to form plaques at both the permissive and non-permissive temperatures. Thus it is possible to measure the growth of the wild-type virus in the presence of the cold adapted virus by comparing the virus yield at the non-permissive temperature of the cells singly infected with wild-type virus to the yield at the non-permissive temperature of the wild-type virus in doubly infected cells.

Cold-adapted equine influenza viruses of the present invention are characterized primarily by one or more of the following identifying phenotypes: cold-adaptation, temperature sensitivity, dominant interference, and/or attenuation. As used herein, the phrase "an equine influenza virus comprises the identifying phenotype(s) of cold-adaptation,

temperature sensitivity, dominant interference, and/or attenuation" refers to a virus having such a phenotype(s). Examples of such viruses include, but are not limited to, EIV-P821, identified by accession No. ATCC VR-2625, EIV-P824, identified by accession No. ATCC VR-2624, and EIV-MSV+5, identified by accession No. ATCC VR-2627, as well as EIV-MSV0, EIV, MSV+1, EIV-MSV+2, EIV-MSV+3, and EIV-MSV+4. Production of such viruses is described in the examples. For example, cold-adapted equine influenza virus EIV-P821 is characterized by, i.e., has the identifying phenotypes of, (a) cold-adaptation, e.g., its ability to replicate in embryonated chicken eggs at a temperature of about 26°C; (b) temperature sensitivity, e.g., its inability to form plaques in tissue culture cells and to express late gene products at a non-permissive temperature of about 37°C, and its inability to form plaques in tissue culture cells and to synthesize any viral proteins at a non-permissive temperature of about 39°C; (c) its attenuation upon administration to an equine influenza virus-susceptible animal; and (d) dominant interference, e.g., its ability, when co-infected into a cell with a wild-type influenza A virus, to interfere with the growth of that wild-type virus. Similarly, cold-adapted equine influenza virus EIV-P824 is characterized by (a) cold adaptation, e.g., its ability to replicate in embryonated chicken eggs at a temperature of about 28°C; (b) temperature sensitivity, e.g., its inability to form plaques in tissue culture cells at a non-permissive temperature of about 39°C; and (c) dominant interference, e.g., its ability, when co-infected into a cell with a wild-type influenza A virus, to interfere with the growth of that wild-type virus. In another example, cold-adapted equine influenza virus EIV-MSV+5 is characterized by (a) cold-adaptation, e.g., its ability to replicate in embryonated chicken eggs at a temperature of about 26°C; (b) temperature sensitivity, e.g., its inability to form plaques in tissue culture cells at a non-permissive temperature of about

39°C; and (c) its attenuation upon administration to an equine influenza virus-susceptible animal.

In certain cases, the RNA segment upon which one or more mutations associated with a certain phenotype occur may be determined through reassortment analysis by standard methods, as disclosed herein. In one embodiment, a cold-adapted equine influenza virus of the present invention comprises a temperature sensitive phenotype that correlates with at least two mutations in the genome of that virus. In this embodiment, one of the two mutations, localized by reassortment analysis as disclosed herein, inhibits, i.e., blocks or prevents, the ability of the virus to form plaques in tissue culture cells at a non-permissive temperature of about 39°C. This mutation co-segregates with the segment of the equine influenza virus genome that encodes the nucleoprotein (NP) gene of the virus, i.e., the mutation is located on the same RNA segment as the NP gene. In this embodiment, the second mutation inhibits all protein synthesis at a non-permissive temperature of about 39°C. As such, at the non-permissive temperature, the virus genome is incapable of expressing any viral proteins. Examples of cold-adapted equine influenza viruses possessing these characteristics are EIV-P821 and EIV MSV+5. EIV-P821 was generated by serial passaging of a wild-type equine influenza virus in embryonated chicken eggs by methods described in Example 1A. EIV-MSV+5 was derived by further serial passaging of EIV-P821, as described in Example 1E.

Furthermore, a cold-adapted, temperature sensitive equine influenza virus comprising the two mutations which inhibit plaque formation and viral protein synthesis at a non-permissive temperature of about 39°C can comprise one or more additional mutations, which inhibit the virus' ability to synthesize late gene products and to form plaques in tissue culture cells at a non-permissive temperature of about 37°C. An example of a cold-adapted equine

influenza virus possessing these characteristics is EIV-P821. This virus isolate replicates in embryonated chicken eggs at a temperature of about 26°C, and does not form plaques or express any viral proteins at a temperature of about 39°C. Furthermore, EIV-P821 does not form plaques on MDCK cells at a non-permissive temperature of about 37°C, and at this temperature, late gene expression is inhibited in such a way that late proteins are not produced, i.e., normal levels of NP protein are synthesized, reduced or undetectable levels of M1 or HA proteins are synthesized, and enhanced levels of the polymerase proteins are synthesized. Since this phenotype is typified by differential viral protein synthesis, it is distinct from the protein synthesis phenotype seen at a non-permissive temperature of about 39°C, which is typified by the inhibition of synthesis of all viral proteins.

Pursuant to 37 CFR § 1.802 (a-c), cold-adapted equine influenza viruses, designated herein as EIV-P821, an EIV-P824 were deposited with the American Type Culture Collection (ATCC, 10801 University Boulevard, Manassas, VA 20110-2209) under the Budapest Treaty as ATCC Accession Nos. ATCC VR-2625, and ATCC VR-2624, respectively, on July 11, 1998. Cold-adapted equine influenza virus EIV-MSV+5 was deposited with the ATCC as ATCC Accession No. ATCC VR-2627on August 3, 1998. Pursuant to 37 CFR§ 1.806, the deposits are made for a term of at least thirty (30) years and at least five (5) years after the most recent request for the furnishing of a sample of the deposit was received by the depository. Pursuant to 37 CFR § 1.808 (a)(2), all restrictions imposed by the depositor on the availability to the public will be irrevocably removed upon the granting of the patent.

Preferred cold-adapted equine influenza viruses of the present invention have the identifying phenotypes of EIV-P821, EIV-P824, and EIV-MSV+5. Particularly preferred cold-adapted equine influenza viruses include EIV-P821, EIV-P824, EIV-MSV+5, and

progeny of these viruses. As used herein, "progeny" are "offspring," and as such can slightly altered phenotypes compared to the parent virus, but retain identifying phenotypes of the parent virus, for example, cold-adaptation, temperature sensitivity, dominant interference, or attenuation. For example, cold-adapted equine influenza virus EIV-MSV+5 is a "progeny" of cold-adapted equine influenza virus EIV-P821. "Progeny" also include reassortant influenza A viruses that comprise one or more identifying phenotypes of the donor parent virus.

Reassortant influenza A viruses of the present invention are produced by genetic reassortment of the genome segments of a donor cold-adapted equine influenza virus of the present invention with the genome segments of a recipient influenza A virus, and then selecting a reassortant virus that derives at least one of its eight RNA genome segments from the donor virus, such that the reassortant virus acquires at least one identifying phenotype of the donor cold-adapted equine influenza virus. Identifying phenotypes include cold-adaptation, temperature sensitivity, attenuation, and dominant interference. Preferably, reassortant influenza A viruses of the present invention derive at least the attenuation phenotype of the donor virus. Methods to isolate reassortant influenza viruses are well known to those skilled in the art of virology and are disclosed, for example, in Fields, et al., 1996, *Fields Virology, 3d ed.*, Lippincott-Raven; and Palese, et al., 1976, *J. Virol.*, *17*, 876-884. Fields, et al., *ibid.* and Palese, et al., *ibid.*

A suitable donor equine influenza virus is a cold-adapted equine influenza virus of the present invention, for example, EIV-P821, identified by accession No. ATCC VR-2625, EIV-P824, identified by accession No. ATCC VR-2624, or EIV-MSV+5, identified by accession No. ATCC VR-2627. A suitable recipient influenza A virus can be another equine influenza virus, for example a Eurasian subtype 2 equine influenza virus such as

A/equine/Suffolk/89 (H3N8) or a subtype 1 equine influenza virus such as A/Prague/1/56 (H7N7). A recipient influenza A virus can also be any influenza A virus capable of forming a reassortant virus with a donor cold-adapted equine influenza virus. Examples of such influenza A viruses include, but are not limited to, human influenza viruses such as A/Puerto Rico/8/34 (H1N1), A/Hong Kong/156/97 (H5N1), A/Singapore/1/57 (H2N2), and A/Hong Kong/1/68 (H3N2); swine viruses such as A/Swine/Iowa/15/30 (H1N1); and avian viruses such as A/mallard/New York/6750/78 (H2N2) and A/chicken/Hong Kong/258/97 (H5N1). A reassortant virus of the present invention can include any combination of donor and recipient gene segments, as long as the resulting reassortant virus possesses at least one identifying phenotype of the donor virus.

One example of a reassortant virus of the present invention is a "6 + 2" reassortant virus, in which the six "internal gene segments," i.e., those comprising the NP, PB2, PB1, PA, M, and NS genes, are derived from the donor cold-adapted equine influenza virus genome, and the two "external gene segments," i.e., those comprising the HA and NA genes, are derived from the recipient influenza A virus. A resultant virus thus produced has the attenuated, cold-adapted, temperature sensitive, and/or dominant interference phenotypes of the donor cold-adapted equine influenza virus, but the antigenicity of the recipient strain.

In yet another embodiment, a cold-adapted equine influenza virus of the present invention can be produced through recombinant means. In this approach, one or more specific mutations, associated with identified cold-adaptation, attenuation, temperature sensitivity, or dominant interference phenotypes, are identified and are introduced back into a wild-type equine influenza virus strain using a reverse genetics approach. Reverse genetics entails using RNA polymerase complexes isolated from influenza virus-infected cells to transcribe artificial influenza virus genome segments containing the mutation(s),

incorporating the synthesized RNA segment(s) into virus particles using a helper virus, and then selecting for viruses containing the desired changes. Reverse genetics methods for influenza viruses are described, for example, in Enami, et al., 1990, *Proc. Natl. Acad. Sci. 87*, 3802-3805; and in U.S. Patent No. 5,578,473, by Palese, et al., issued November 26, 1996. This approach allows one skilled in the art to produce additional cold-adapted equine influenza viruses of the present invention without the need to go through the lengthy cold-adaptation process, and the process of selecting mutants both *in vitro* and *in vivo* with the desired virus phenotype.

A cold-adapted equine influenza virus of the present invention may be propagated by standard virological methods well-known to those skilled in the art, examples of which are disclosed herein. For example, a cold-adapted equine influenza virus can be grown in embryonated chicken eggs or in eukaryotic tissue culture cells. Suitable continuous eukaryotic cell lines upon which to grow a cold-adapted equine influenza virus of the present invention include those that support growth of influenza viruses, for example, MDCK cells. Other suitable cells upon which to grow a cold-adapted equine influenza virus of the present invention include, but are not limited to, primary kidney cell cultures of monkey, calf, hamster or chicken.

In one embodiment, the present invention provides a therapeutic composition to protect an animal against disease caused by an influenza A virus, where the therapeutic composition includes either a cold-adapted equine influenza virus or a reassortant influenza A virus comprising at least one genome segment of an equine influenza virus generated by cold-adaptation, wherein the equine influenza virus genome segment confers at least one identifying phenotype of the cold-adapted equine influenza virus. In addition, a therapeutic composition of the present invention can include an equine influenza virus that has been

genetically engineered to comprise one or more mutations, where those mutations have been identified to confer a certain identifying phenotype on a cold-adapted equine influenza virus of the present invention. As used herein, the phrase "disease caused by an influenza A virus" refers to the clinical manifestations observed in an animal which has been infected with a virulent influenza A virus. Examples of such clinical manifestations include, but are not limited to, fever, sneezing, coughing, nasal discharge, rales, anorexia and depression. In addition, the phrase "disease caused by an influenza A virus" is defined herein to include shedding of virulent virus by the infected animal. Verification that clinical manifestations observed in an animal correlate with infection by virulent equine influenza virus may be made by several methods, including the detection of a specific antibody and/or T-cell responses to equine influenza virus in the animal. Preferably, verification that clinical manifestations observed in an animal correlate with infection by a virulent influenza A virus is made by the isolation of the virus from the afflicted animal, for example, by swabbing the nasopharyngeal cavity of that animal for virus-containing secretions. Verification of virus isolation may be made by the detection of CPE in tissue culture cells inoculated with the isolated secretions, by inoculation of the isolated secretions into embryonated chicken eggs, where virus replication is detected by the ability of AF from the inoculated eggs to agglutinate erythrocytes, suggesting the presence of the influenza virus hemagglutinin protein, or by use of a commercially available diagnostic test, for example, the Directigen® FLU A test.

As used herein, the term "to protect" includes, for example, to prevent or to treat influenza A virus infection in the subject animal. As such, a therapeutic composition of the present invention can be used, for example, as a prophylactic vaccine to protect a subject

animal from influenza disease by administering the therapeutic composition to that animal at some time prior to that animal's exposure to the virulent virus.

A therapeutic composition of the present invention, comprising a cold-adapted equine influenza virus having a dominant interference phenotype, can also be used to treat an animal that has been recently infected with virulent influenza A virus or is likely to be subsequently exposed in a few days, such that the therapeutic composition immediately interferes with the growth of the virulent virus, prior to the animal's production of antibodies to the virulent virus. A therapeutic composition comprising a cold-adapted equine influenza virus having a dominant interference phenotype may be effectively administered prior to subsequent exposure for a length of time corresponding to the approximate length of time that a cold-adapted equine influenza virus of the present invention will replicate in the upper respiratory tract of a treated animal, for example, up to about seven days. A therapeutic composition comprising a cold-adapted equine influenza virus having a dominant interference phenotype may be effectively administered following exposure to virulent equine influenza virus for a length of time corresponding to the time required for an infected animal to show disease symptoms, for example, up to about two days.

Therapeutic compositions of the present invention can be administered to any animal susceptible to influenza virus disease, for example, humans, swine, horses and other equids, aquatic birds, domestic and game fowl, seals, mink, and whales. Preferably, a therapeutic composition of the present invention is administered equids. Even more preferably, a therapeutic composition of the present invention is administered to a horse, to protect against equine influenza disease.

Current vaccines available to protect horses against equine influenza virus disease are not effective in protecting young foals, most likely because they cannot overcome the

maternal antibody present in these young animals, and often, vaccination at an early age, for example 3 months of age, can lead to tolerance rather than immunity. In one embodiment, and in contrast to existing equine influenza virus vaccines, a therapeutic composition comprising a cold-adapted equine influenza virus of the present invention apparently can produce immunity in young animals. As such, a therapeutic composition of the present invention can be safely and effectively administered to young foals, as young as about 3 months of age, to protect against equine influenza disease without the induction of tolerance.

In one embodiment, a therapeutic composition of the present invention can be multivalent. For example, it can protect an animal from more than one strain of influenza A virus by providing a combination of one or more cold-adapted equine influenza viruses of the present invention, one or more reassortant influenza A viruses, and/or one or more genetically-engineered equine influenza viruses of the present invention. Multivalent therapeutic compositions can include at least two cold-adapted equine influenza viruses, e.g., against North American subtype-2 virus isolates such as A/equine/Kentucky/1/91 (H1N8), and Eurasian subtype-2 virus isolates such as A/equine/Suffolk/89 (H3N8); or one or more subtype-2 virus isolates and a subtype-1 virus isolate such as A/equine/Prague/1/56 (H7N7). Similarly, a multivalent therapeutic composition of the present invention can include a cold-adapted equine influenza virus and a reassortant influenza A virus of the present invention, or two reassortant influenza A viruses of the present invention. A multivalent therapeutic composition of the present invention can also contain one or more formulations to protect against one or more other infectious agents in addition to influenza A virus. Such other infectious agents include, but not limited to: viruses; bacteria; fungi and fungal-related microorganisms; and parasites. Preferable multivalent therapeutic compositions include, but are not limited to, a cold-adapted equine influenza virus, reassortant influenza A virus, or

genetically-engineered equine influenza virus of the present invention plus one or more compositions protective against one or more other infectious agents that afflict horses. Suitable infectious agents to protect against include, but are not limited to, equine infectious anemia virus, equine herpes virus, eastern, western, or Venezuelan equine encephalitis virus, tetanus, *Streptococcus equi*, and *Ehrlichia resticii*.

A therapeutic composition of the present invention can be formulated in an excipient that the animal to be treated can tolerate. Examples of such excipients include water, saline, Ringer's solution, dextrose solution, Hank's solution, and other aqueous physiologically balanced salt solutions. Excipients can also contain minor amounts of additives, such as substances that enhance isotonicity and chemical or biological stability. Examples of buffers include phosphate buffer, bicarbonate buffer, and Tris buffer, while examples of stabilizers include A1/A2 stabilizer, available from Diamond Animal Health, Des Moines, IA. Standard formulations can either be liquids or solids which can be taken up in a suitable liquid as a suspension or solution for administration to an animal. In one embodiment, a non-liquid formulation may comprise the excipient salts, buffers, stabilizers, etc., to which sterile water or saline can be added prior to administration.

A therapeutic composition of the present invention may also include one or more adjuvants or carriers. Adjuvants are typically substances that enhance the immune response of an animal to a specific antigen, and carriers include those compounds that increase the half-life of a therapeutic composition in the treated animal. One advantage of a therapeutic composition comprising a cold-adapted equine influenza virus or a reassortant influenza A virus of the present invention is that adjuvants and carriers are not required to produce an efficacious vaccine. Furthermore, in many cases known to those skilled in the art, the advantages of a therapeutic composition of the present invention would be hindered by the

use of some adjuvants or carriers. However, it should be noted that use of adjuvants or carriers is not precluded by the present invention.

Therapeutic compositions of the present invention include an amount of a cold-adapted equine influenza virus that is sufficient to protect an animal from challenge with virulent equine influenza virus. In one embodiment, a therapeutic composition of the present invention can include an amount of a cold-adapted equine influenza virus ranging from about 10^5 tissue culture infectious dose-50 ($TCID_{50}$) units of virus to about 10^8 $TCID_{50}$ units of virus. As used herein, a "$TCID_{50}$ unit" is amount of a virus which results in cytopathic effect in 50% of those cell cultures infected. Methods to measure and calculate $TCID_{50}$ are known to those skilled in the art and are available, for example, in Reed and Muench, 1938, *Am. J. of Hyg. 27*, 493-497. A preferred therapeutic composition of the present invention comprises from about 10^6 $TCID_{50}$ units to about 10^7 $TCID_{50}$ units of a cold-adapted equine influenza virus or reassortant influenza A virus of the present invention. Even more preferred is a therapeutic composition comprising about 2 x 10^6 $TCID_{50}$ units of a cold-adapted equine influenza virus or reassortant influenza A virus of the present invention.

The present invention also includes methods to protect an animal against disease caused by an influenza A virus comprising administering to the animal a therapeutic composition of the present invention. Preferred are those methods which protect an equid against disease caused by equine influenza virus, where those methods comprise administering to the equid a cold-adapted equine influenza virus. Acceptable protocols to administer therapeutic compositions in an effective manner include individual dose size, number of doses, frequency of dose administration, and mode of administration. Determination of such protocols can be accomplished by those skilled in the art, and examples are disclosed herein.

A preferable method to protect an animal against disease caused by an influenza A virus includes administering to that animal a single dose of a therapeutic composition comprising a cold-adapted equine influenza virus, a reassortant influenza A virus, or genetically-engineered equine influenza virus of the present invention. A suitable single dose is a dose that is capable of protecting an animal from disease when administered one or more times over a suitable time period. The method of the present invention may also include administering subsequent, or booster doses of a therapeutic composition. Booster administrations can be given from about 2 weeks to several years after the original administration. Booster administrations preferably are administered when the immune response of the animal becomes insufficient to protect the animal from disease. Examples of suitable and preferred dosage schedules are disclosed in the Examples section.

A therapeutic composition of the present invention can be administered to an animal by a variety of means, such that the virus will enter and replicate in the mucosal cells in the upper respiratory tract of the treated animal. Such means include, but are not limited to, intranasal administration, oral administration, and intraocular administration. Since influenza viruses naturally infect the mucosa of the upper respiratory tract, a preferred method to administer a therapeutic composition of the present invention is by intranasal administration. Such administration may be accomplished by use of a syringe fitted with cannula, or by use of a nebulizer fitted over the nose and mouth of the animal to be vaccinated.

The efficacy of a therapeutic composition of the present invention to protect an animal against disease caused by influenza A virus can be tested in a variety of ways including, but not limited to, detection of antibodies by, for example, hemagglutination inhibition (HAI) tests, detection of cellular immunity within the treated animal, or challenge of the treated animal with virulent equine influenza virus to determine whether the treated

animal is resistant to the development of disease. In addition, efficacy of a therapeutic composition of the present invention comprising a cold-adapted equine influenza virus having a dominant interference phenotype to ameliorate or reduce disease symptoms in an animal previously inoculated or susceptible to inoculation with a virulent, wild-type equine influenza virus can be tested by screening for the reduction or absence of disease symptoms in the treated animal.

The present invention also includes methods to produce a therapeutic composition of the present invention. Suitable and preferred methods for making a therapeutic composition of the present invention are disclosed herein. Pertinent steps involved in producing one type of therapeutic composition of the present invention, i.e., a cold-adapted equine influenza virus, include (a) passaging a wild-type equine influenza virus *in vitro*, for example, in embryonated chicken eggs; (b) selecting viruses that grow at a reduced temperature; (c) repeating the passaging and selection steps one or more times, at progressively lower temperatures, until virus populations are selected which stably grow at the desired lower temperature; and (d) mixing the resulting virus preparation with suitable excipients.

The pertinent steps involved in producing another type of therapeutic composition of the present invention, i.e., a reassortant influenza A virus having at least one genome segment of an equine influenza virus generated by adaptation, includes the steps of (a) mixing the genome segments of a donor cold-adapted equine influenza virus, which preferably also has the phenotypes of attenuation, temperature sensitivity, or dominant interference, with the genome segments of a recipient influenza A virus, and (b) selecting reassortant viruses that have at least one identifying phenotype of the donor equine influenza virus. Identifying phenotypes to select for include attenuation, cold-adaptation, temperature sensitivity, and dominant interference. Methods to screen for these phenotypes are well

known to those skilled in the art, and are disclosed herein. It is preferable to screen for viruses that at least have the phenotype of attenuation.

Using this method to generate a reassortant influenza A virus having at least one genome segment of a equine influenza virus generated by cold-adaptation, one type of reassortant virus to select for is a "6 + 2" reassortant, where the six "internal gene segments," i.e., those coding for the NP, PB2, PB1, PA, M, and NS genes, are derived from the donor cold-adapted equine influenza virus genome, and the two "external gene segments," i.e., those coding for the HA and NA genes, are derived from the recipient influenza A virus. A resultant virus thus produced can have the cold-adapted, attenuated, temperature sensitive, and/or interference phenotypes of the donor cold-adapted equine influenza virus, but the antigenicity of the recipient strain.

The present invention includes nucleic acid molecules isolated from equine influenza virus wild type strain A/equine/Kentucky/1/91 (H3N8), and cold-adapted equine influenza virus EIV-P821.

In accordance with the present invention, an isolated nucleic acid molecule is a nucleic acid molecule that has been removed from its natural milieu (i.e., that has been subject to human manipulation) and can include DNA, RNA, or derivatives of either DNA or RNA. As such, "isolated" does not reflect the extent to which the nucleic acid molecule has been purified.

The present invention includes nucleic acid molecules encoding wild-type and cold-adapted equine influenza virus proteins. Nucleic acid molecules of the present invention can be prepared by methods known to one skilled in the art. Proteins of the present invention can be prepared by methods known to one skilled in the art, *i.e.*, recombinant DNA technology. Preferred nucleic acid molecules have coding strands comprising nucleic acid sequences

SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO:54, SEQ ID NO: 56, SEQ ID NO:57, SEQ ID NO:59, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO:70, SEQ ID NO: 71, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO:82 ,SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 90, SEQ ID NO: 91, SEQ ID NO: 93, SEQ ID NO: 94, SEQ ID NO: 96, SEQ ID NO:103, SEQ ID NO: 105, SEQ ID NO: 106 and SEQ ID NO: 108 and/or a complement thereof. Complements are defined as two single strands of nucleic acid in which the nucleotide sequence is such that they will hybridize as a result of base pairing throughout their full length. Given a nucleotide sequence, one of ordinary skill in the art can deduce the complement.

Preferred nucleic acid molecules encoding equine influenza M proteins are $nei_{wt}M_{1023}$, $nei_{wt1}M_{1023}$, $nei_{wt2}M_{1023}$, $nei_{wt}M_{756}$, $nei_{wt1}M_{756}$, $nei_{wt2}M_{756}$, $nei_{ca1}M_{1023}$, $nei_{ca2}M_{1023}$, $nei_{ca1}M_{756}$, and/or $nei_{ca2}M_{756}$, the coding strands of which are represented by SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, and/or SEQ ID NO:6.

Preferred nucleic acid molecules encoding equine influenza HA proteins are $nei_{wt}HA_{1762}$, $nei_{wt}HA_{1695}$, $nei_{ca1}HA_{1762}$, $nei_{ca2}HA_{1762}$, $nei_{ca1}HA_{1695}$, and/or $nei_{ca2}HA_{1695}$, the coding strands of which are represented by SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, and/or SEQ ID NO:12.

Preferred nucleic acid molecules encoding equine influenza PB2-N proteins are nei_{wt}PB2-N_{1241}, nei_{wt}PB2-N_{1214}, nei_{ca1}PB2-N_{1241} nei_{ca2}PB2-N_{1241}, nei_{ca1}PB2-N_{1214} nei_{ca2},

and/or PB2-N_{1214}, the coding strands of which are represented by SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:16, and/or SEQ ID NO:18.

Preferred nucleic acid molecules encoding equine influenza PB2-C proteins are nei_{wt1}PB2-C_{1233}, nei_{wt2}PB2-C_{1232}, nei_{wt}PB2-C_{1194}, nei_{ca1}PB2-C_{1232}, nei_{ca2}PB2-C_{1231}, and/or nei_{ca1}PB2-C_{1194}, the coding strands of which are represented by SEQ ID NO:19, SEQ ID NO:22, SEQ ID NO:21, SEQ ID NO:23, and/or SEQ ID NO:25.

Preferred nucleic acid molecules encoding equine influenza PB2 proteins are nei_{wt}PB2$_{2341}$, nei_{wt}PB2$_{2277}$, nei_{ca1}PB2$_{2341}$, and/or nei_{ca1}PB2$_{2277}$, the coding strands of which are represented by SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:47, and/or SEQ ID NO:49.

Preferred nucleic acid molecules encoding equine influenza NS proteins are nei_{wt1}NS$_{891}$, nei_{wt2}NS$_{891}$, nei_{wt1}NS$_{690}$, nei_{wt2}NS$_{690}$, nei_{wt3}NS$_{888}$, nei_{wt3}NS$_{690}$, nei_{wt4}NS$_{468}$, nei_{wt4}NS$_{293}$, nei_{ca1}NS$_{888}$, nei_{ca2}NS$_{888}$, nei_{ca1}NS$_{690}$, and/or nei_{ca2}NS$_{690}$the coding strands of which are represented by SEQ ID NO:50, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:56, SEQ ID NO:57 and/or SEQ ID NO:59.

Preferred nucleic acid molecules encoding equine influenza PB1-N proteins are nei_{wt1}PB1-N_{1229}, nei_{wt1}PB1N_{1194}, nei_{wt2}PB1-N_{673}, nei_{wt2}PB1-N_{636}, nei_{ca1}PB1-N_{1225}, nei_{ca1}PB1-N_{1185}, nei_{ca2}PB1-N_{1221}, and/or nei_{ca2}PB1-N_{1185} the coding strands of which are represented by SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:70, and/or SEQ ID NO:71.

Preferred nucleic acid molecules encoding equine influenza PA-C proteins are nei_{wt1}PA-C_{1228}, nei_{wt1}PA-C_{1164}, nei_{wt2}PA-C_{1223}, nei_{wt2}PA-C_{1164}, nei_{ca1}PA-C_{1233}, nei_{ca2}PA-C_{1233}, nei_{ca1}PA-C_{1170}, and/or nei_{ca2}PA-C_{1170} the coding strands of which are represented by SEQ ID NO:76, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, and/or SEQ ID NO:82.

Preferred nucleic acid molecules encoding equine influenza PB1-C proteins are $nei_{wt1}PB1\text{-}C_{1234}$, $nei_{wt1}PB1\text{-}C_{1188}$, $nei_{wt2}PB1\text{-}C_{1240}$, $nei_{wt2}PB1\text{-}C_{1188}$, $nei_{ca1}PB1\text{-}C_{1241}$, $nei_{ca1}PB1\text{-}C_{1188}$, $nei_{ca2}PB1\text{-}C_{1241}$and/or $nei_{ca2}PB1\text{-}C_{1188}$, the coding strands of which are represented by SEQ ID NO:85, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:93, SEQ ID NO:94and/or SEQ ID NO:96.

Preferred nucleic acid molecules encoding equine influenza PB1 proteins are $nei_{wt}PB1_{2341}$, $nei_{wt}PB1_{2271}$, $nei_{ca1}PB1_{2341}$, $nei_{ca1}PB1_{2271}$, the coding strands of which are represented by SEQ ID NO:103, SEQ ID NO:105, SEQ ID NO:106, and/or SEQ ID NO:108.

The present invention includes proteins comprising SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:14, SEQ ID NO:17, SEQ ID NO:20, SEQ ID NO:24, SEQ ID NO:45, SEQ ID NO:48, SEQ ID NO:51, SEQ ID NO: 55, SEQ ID NO:58, SEQ ID NO: 63, SEQ ID NO:66, SEQ ID NO: 69, SEQ ID NO: 77, SEQ ID NO: 81, SEQ ID NO:86, SEQ ID NO: 89, SEQ ID NO: 92, SEQ ID NO:95, SEQ ID NO:104 and SEQ ID NO: 107 as well as nucleic acid molecules encoding such proteins.

Preferred equine influenza M proteins of the present invention include proteins encoded by a nucleic acid molecule comprising $nei_{wt}M_{1023}$, $nei_{wt1}M_{1023}$, $nei_{wt2}M_{1023}$, $nei_{wt}M_{756}$, $nei_{wt1}M_{756}$, $nei_{wt2}M_{756}$, $nei_{ca1}M_{1023}$, $nei_{ca2}M_{1023}$, $nei_{ca1}M_{756}$, and/or $nei_{ca2}M_{756}$. Preferred equine influenza M proteins are $Pei_{wt}M_{252}$, $Pei_{ca1}M_{252}$, and/or $Pei_{ca2}M_{252}$. In one embodiment, a preferred equine influenza M protein of the present invention is encoded by SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, and/or SEQ ID NO:6, and, as such, has an amino acid sequence that includes SEQ ID NO:2 and/or SEQ ID NO:5.

Preferred equine influenza HA proteins of the present invention include proteins encoded by a nucleic acid molecule comprising $nei_{wt}HA_{1762}$, $nei_{wt}HA_{1695}$, $nei_{ca1}HA_{1762}$, $nei_{ca2}HA_{1762}$, $nei_{ca1}HA_{1695}$, and/or $nei_{ca2}HA_{1695}$. Preferred equine influenza HA proteins are

P $Pei_{wt}HA_{565}$, $Pei_{ca1}HA_{565}$, and/or $Pei_{ca2}HA_{565}$. In one embodiment, a preferred equine influenza HA protein of the present invention is encoded by SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, and/or SEQ ID NO:12, and, as such, has an amino acid sequence that includes SEQ ID NO:8 and/or SEQ ID NO:11.

Preferred equine influenza PB2-N proteins of the present invention include proteins encoded by a nucleic acid molecule comprising $nei_{wt}PB2\text{-}N_{1241}$, $nei_{wt}PB2\text{-}N_{1214}$, $nei_{ca1}PB2\text{-}N_{1241}$ $nei_{ca2}PB2\text{-}N_{1241}$, $nei_{ca1}PB2\text{-}N_{1214}$ nei_{ca2}, and/or $PB2\text{-}N_{1214}$. Preferred equine influenza PB2-N proteins are $P_{wt}PB2\text{-}N_{404}$, $P_{ca1}PB2\text{-}N_{404}$, and/or $P_{ca2}PB2\text{-}N_{404}$. In one embodiment, a preferred equine influenza PB2-N protein of the present invention is encoded by SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:16, and/or SEQ ID NO:18, and, as such, has an amino acid sequence that includes SEQ ID NO:14 and/or SEQ ID NO:17.

Preferred equine influenza PB2-C proteins of the present invention include proteins encoded by a nucleic acid molecule comprising $nei_{wt1}PB2\text{-}C_{1233}$, $nei_{wt2}PB2\text{-}C_{1232}$, $nei_{wt}PB2\text{-}C_{1194}$, $nei_{ca1}PB2\text{-}C_{1232}$, $nei_{ca2}PB2\text{-}C_{1231}$, and/or $nei_{ca1}PB2\text{-}C_{1194}$. Preferred equine influenza PB2-N proteins are $P_{wt}PB2\text{-}C_{398}$, $P_{ca1}PB2\text{-}C_{398}$, and/or $P_{ca2}PB2\text{-}C_{398}$. In one embodiment, a preferred equine influenza PB2-C protein of the present invention is encoded by SEQ ID NO:19, SEQ ID NO:22, SEQ ID NO:21, SEQ ID NO:23, and/or SEQ ID NO:25, and, as such, has an amino acid sequence that includes SEQ ID NO:20 and/or SEQ ID NO:24.

Preferred equine influenza PB2 proteins of the present invention include proteins encoded by a nucleic acid molecule comprising $nei_{wt}PB2_{2341}$, $nei_{wt}PB2_{2277}$, $nei_{ca1}PB2_{2341}$, and or $nei_{ca1}PB2_{2277}$. Preferred equine influenza PB2 proteins are $Pei_{wt}PB2_{759}$,and/or $Pei_{ca1}PB2_{759}$. In one embodiment, a preferred equine influenza PB2 protein of the present invention is encoded by SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:47, and/or SEQ ID

NO:49, and, as such, has an amino acid sequence that includes SEQ ID NO:45 and/or SEQ ID NO:48.

Preferred equine influenza NS proteins of the present invention include proteins encoded by a nucleic acid molecule comprising $nei_{wt1}NS_{891}$, $nei_{wt2}NS_{891}$, $nei_{wt1}NS_{690}$, $nei_{wt3}NS_{888}$, $nei_{wt4}NS_{468}$, $nei_{wt4}NS_{293}$, $nei_{ca1}NS_{888}$, $nei_{ca2}NS_{888}$, and/or $nei_{ca1}NS_{690}$. Preferred equine influenza NS proteins are $Pei_{wt}NS_{230}$, $Pei_{wt4}NS_{97}$, and/or $Pei_{ca1}NS_{230}$. In one embodiment, a preferred equine influenza NS protein of the present invention is encoded by SEQ ID NO:50, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:56, SEQ ID NO:57 and/or SEQ ID NO:59, and, as such, has an amino acid sequence that includes SEQ ID NO:51, SEQ ID NO:55 and/or SEQ ID NO:58.

Preferred equine influenza PB1-N proteins of the present invention include proteins encoded by a nucleic acid molecule comprising nei_{wt1}PB1-N_{1229}, nei_{wt1}PB1N_{1194}, nei_{wt2}PB1-N_{673}, nei_{wt2}PB1-N_{636}, nei_{ca1}PB21-N_{1225}, nei_{ca1}PB1-N_{1185}, and/or nei_{ca2}PB1-N_{1221}. Preferred equine influenza PB1-N proteins are Pei_{wt1}PB1-N_{398}, P_{wt2}PB1-N_{212}, and/or P_{ca1}PB1-N_{395}. In one embodiment, a preferred equine influenza PB1-N protein of the present invention is encoded by SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:70, and/or SEQ ID NO:71, and, as such, has an amino acid sequence that includes SEQ ID NO:63, SEQ ID NO:66 and/or SEQ ID NO:69.

Preferred equine influenza PB1-C proteins of the present invention include proteins encoded by a nucleic acid molecule comprising nei_{wt1}PB1-C_{1234}, nei_{wt1}PB1-C_{1188}, nei_{wt2}PB1-C_{1240}, nei_{wt2}PB1-C_{1188}, nei_{ca1}PB1-C_{1241}, nei_{ca1}PB1-C_{1188}, nei_{ca2}PB1-C_{1241} and/or nei_{ca2}PB1-C_{1188}. Preferred equine influenza PB1-C proteins are Pei_{wt1}PB1-C_{396}, Pei_{wt2}PB1-C_{396} Pei_{ca1}PB1-C_{396}, and/or Pei_{ca2}PB1-C_{396}. In one embodiment, a preferred equine influenza PB1-C protein of the present invention is encoded by SEQ ID NO:85, SEQ ID NO:87, SEQ

ID NO:88, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:93, SEQ ID NO:94, and/or SEQ ID NO:96, and, as such, has an amino acid sequence that includes SEQ ID NO:86,SEQ ID NO:89, SEQ ID NO:92, and/or SEQ ID NO:95.

Preferred equine influenza PB1 proteins of the present invention include proteins encoded by a nucleic acid molecule comprising $nei_{wt}PB1_{2341}$, $nei_{wt}PB1_{2271}$, $nei_{ca1}PB1_{2341}$, $nei_{ca1}PB1_{2271}$. Preferred equine influenza PB1 proteins are $Pei_{wt}PB1_{757}$,and/or $Pei_{ca1}PB1_{757}$. In one embodiment, a preferred equine influenza PB1 protein of the present invention is encoded by SEQ ID NO:103, SEQ ID NO:105, SEQ ID NO:106, and/or SEQ ID NO:108, and, as such, has an amino acid sequence that includes SEQ ID NO:104 and/or SEQ ID NO:107.

Preferred equine influenza PA-C proteins of the present invention include proteins encoded by a nucleic acid molecule comprising $nei_{wt1}PA\text{-}C_{1228}$, $nei_{wt1}PA\text{-}C_{1164}$, $nei_{wt2}PA\text{-}C_{1223}$, $nei_{ca1}PA\text{-}C_{1233}$, $nei_{ca2}PA\text{-}C_{1233}$, and/or $nei_{ca1}PA\text{-}C_{1170}$. Preferred equine influenza PA-C proteins are $Pei_{wt1}PA\text{-}C_{388}$, and/or $Pei_{ca1}PA\text{-}C_{390}$. In one embodiment, a preferred equine influenza PA-C protein of the present invention is encoded by SEQ ID NO:76, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, and/or SEQ ID NO:82, and, as such, has an amino acid sequence that includes SEQ ID NO:77 and/or SEQ ID NO:81.

Nucleic acid sequence SEQ ID NO:1 represents the consensus sequence deduced from the coding strand of PCR amplified nucleic acid molecules denoted herein as $nei_{wt1}M_{1023}$ and $nei_{wt2}M_{1023}$, the production of which is disclosed in the Examples. Nucleic acid sequence SEQ ID NO:4 represents the deduced sequence of the coding strand of PCR amplified nucleic acid molecules denoted herein as $nei_{ca1}M_{1023}$ and $nei_{ca2}M_{1023}$, the production of which is disclosed in the Examples. Nucleic acid sequence SEQ ID NO:7 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid

molecule denoted herein as $nei_{wt}HA_{1762}$, the production of which is disclosed in the Examples. Nucleic acid sequence SEQ ID NO:10 represents the deduced sequence of the coding strand of PCR amplified nucleic acid molecules denoted herein as $nei_{ca1}HA_{1762}$ and $nei_{ca2}HA_{1762}$, the production of which is disclosed in the Examples. Nucleic acid sequence SEQ ID NO:13 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{wt}PB2\text{-}N_{1241}$, the production of which is disclosed in the Examples. Nucleic acid sequence SEQ ID NO:16 represents the deduced sequence of the coding strand of PCR amplified nucleic acid molecules denoted herein as $nei_{ca1}PB2\text{-}N_{1241}$ and $nei_{ca2}PB2\text{-}N_{1241}$, the production of which is disclosed in the Examples. Nucleic acid sequence SEQ ID NO:19 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{wt1}PB2\text{-}C_{1233}$, the production of which is disclosed in the examples. Nucleic acid sequence SEQ ID NO:22 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{wt2}PB2\text{-}C_{1232}$, the production of which is disclosed in the examples. Nucleic acid sequence SEQ ID NO:23 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{ca1}PB2\text{-}C_{1232}$, the production of which is disclosed in the examples. Nucleic acid sequence SEQ ID NO:44 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{wt}PB2_{2341}$, the production of which is disclosed in the Examples. Nucleic acid sequence SEQ ID NO:47 represents the deduced sequence of the coding strand of PCR amplified nucleic acid molecules denoted herein as $nei_{ca1}PB2_{2341}$ the production of which is disclosed in the Examples. Nucleic acid sequence SEQ ID NO:50 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{wt1}NS_{891}$ and $nei_{wt2}NS_{891}$ the production of which is disclosed in the examples. Nucleic acid sequence

SEQ ID NO:53 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{wt3}NS_{888}$, the production of which is disclosed in the examples. Nucleic acid sequence SEQ ID NO:54 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{wt4}NS_{468}$, the production of which is disclosed in the examples. Nucleic acid sequence SEQ ID NO:57 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{ca1}NS_{888}$ and $nei_{ca1}NS_{887}$, the production of which is disclosed in the Examples. Nucleic acid sequence SEQ ID NO:62 represents the deduced sequence of the coding strand of PCR amplified nucleic acid molecules denoted herein as $nei_{wt1}PB1\text{-}N_{1229}$, the production of which is disclosed in the Examples. Nucleic acid sequence SEQ ID NO:65 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{wt2}PB2\text{-}N_{673}$, the production of which is disclosed in the examples. Nucleic acid sequence SEQ ID NO:68 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{ca1}PB1\text{-}N_{1225}$, the production of which is disclosed in the examples. Nucleic acid sequence SEQ ID NO:71 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{ca2}PB1\text{-}N_{1221}$, the production of which is disclosed in the examples. Nucleic acid sequence SEQ ID NO:76 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{wt1}PA\text{-}C_{1228}$, the production of which is disclosed in the examples. Nucleic acid sequence SEQ ID NO:79 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{wt2}PA\text{-}C_{1223}$, the production of which is disclosed in the examples. Nucleic acid sequence SEQ ID NO:80 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{ca1}PA\text{-}C_{1233}$ and

$nei_{ca2}PA\text{-}C_{1233}$ the production of which is disclosed in the examples. Nucleic acid sequence SEQ ID NO:85 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{ca1}PB1\text{-}C_{1234}$ the production of which is disclosed in the examples. Nucleic acid sequence SEQ ID NO:88 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{wt2}PB1\text{-}C_{1240}$ the production of which is disclosed in the examples. Nucleic acid sequence SEQ ID NO:91 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{ca1}PB1\text{-}C_{1241}$ the production of which is disclosed in the examples. Nucleic acid sequence SEQ ID NO:94 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{ca2}PB1\text{-}C_{1241}$ the production of which is disclosed in the examples. Nucleic acid sequence SEQ ID NO:103 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{wt}PB1_{2341}$ the production of which is disclosed in the examples. Nucleic acid sequence SEQ ID NO:105 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{wt}PB1_{2271}$ the production of which is disclosed in the examples. Nucleic acid sequence SEQ ID NO:106 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{ca}PB1_{2341}$ the production of which is disclosed in the examples. Nucleic acid sequence SEQ ID NO:108 represents the deduced sequence of the coding strand of a PCR amplified nucleic acid molecule denoted herein as $nei_{ca}PB1_{2271}$ the production of which is disclosed in the examples. Additional nucleic acid molecules, nucleic acid sequences, proteins and amino acid sequences are described in the Examples.

The present invention includes nucleic acid molecule comprising a cold-adapted equine influenza virus encoding an M protein having an amino acid sequence comprising

SEQ ID NO:5. Another embodiment of the present invention includes a nucleic acid molecule comprising a cold-adapted equine influenza virus encoding an HA protein having an amino acid sequence comprising SEQ ID NO:11. Another embodiment of the present invention includes a nucleic acid molecule comprising a cold-adapted equine influenza virus encoding a PB2-N protein having an amino acid sequence comprising SEQ ID NO:17. Another embodiment of the present invention includes a nucleic acid molecule comprising a cold-adapted equine influenza virus encoding a PB2-C protein having an amino acid sequence comprising SEQ ID NO:24. Another embodiment of the present invention includes a nucleic acid molecule comprising a cold-adapted equine influenza virus encoding a PB protein having an amino acid sequence comprising SEQ ID NO:48. Another embodiment of the present invention includes a nucleic acid molecule comprising a cold-adapted equine influenza virus encoding a NS protein having an amino acid sequence comprising SEQ ID NO:58. Another embodiment of the present invention includes a nucleic acid molecule comprising a cold-adapted equine influenza virus encoding a PB1-N protein having an amino acid sequence comprising SEQ ID NO:69. Another embodiment of the present invention includes a nucleic acid molecule comprising a cold-adapted equine influenza virus encoding a PA-C protein having an amino acid sequence comprising SEQ ID NO:81. Another embodiment of the present invention includes a nucleic acid molecule comprising a cold-adapted equine influenza virus encoding a PB1-C protein having an amino acid sequence comprising SEQ ID NO:92. Another embodiment of the present invention includes a nucleic acid molecule comprising a cold-adapted equine influenza virus encoding a PB1 protein having an amino acid sequence comprising SEQ ID NO:107.

It should be noted that since nucleic acid sequencing technology is not entirely error-free, the nucleic acid sequences and amino acid sequences presented herein represent,

respectively, apparent nucleic acid sequences of nucleic acid molecules of the present invention and apparent amino acid sequences of M, HA, PB2-N, PB2-C, PB2, NS, PB1-N, PA-C, PB1-C and PB1 proteins of the present invention.

Another embodiment of the present invention is an antibody that selectively binds to an wild-type virus M, HA, PB2-N, PB2-C, PB2, NS, PB1-N, PA-C, PB1-C and PB1 protein of the present invention. Another embodiment of the present invention is an antibody that selectively binds to a cold-adapted virus M, HA, PB2-N, PB2-C, PB2, NS, PB1-N, PA-C, PB1-C and PB1 protein of the present invention. Preferred antibodies selectively bind to SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:14, SEQ ID NO:17, SEQ ID NO:20, SEQ ID NO:24, SEQ ID NO:45, SEQ ID NO:48, SEQ ID NO:51, SEQ ID NO: 55, SEQ ID NO:58, SEQ ID NO: 63, SEQ ID NO:66, SEQ ID NO: 69, SEQ ID NO: 77, SEQ ID NO: 81, SEQ ID NO:86, SEQ ID NO: 89, SEQ ID NO: 92, SEQ ID NO:95, SEQ ID NO:104 and SEQ ID NO: 107.

The following examples are provided for the purposes of illustration and are not intended to limit the scope of the present invention.

Example 1

This example discloses the production and phenotypic characterization of several cold-adapted equine influenza viruses of the present invention.

A. Parental equine influenza virus, A/equine/Kentucky/1/91 (H3N8) (obtained from Tom Chambers, the University of Kentucky, Lexington, KY) was subjected to cold-adaptation in a foreign host species, i.e., embryonated chicken eggs, in the following manner. Embryonated, 10 or 11-day old chicken eggs, available, for example, from Truslow Farms, Chestertown, MD or from HyVac, Adel, IA, were inoculated with the parental equine influenza virus by injecting about 0.1 milliliter (ml) undiluted AF containing approximately

10^6 plaque forming units (pfu) of virus into the allantoic cavity through a small hole punched in the shell of the egg. The holes in the eggs were sealed with nail polish and the eggs were incubated in a humidified incubator set at the appropriate temperature for three days. Following incubation, the eggs were candled and any non-viable eggs were discarded. AF was harvested from viable embryos by aseptically removing a portion of the egg shell, pulling aside the chorioallantoic membrane (CAM) with sterile forceps and removing the AF with a sterile pipette. The harvested AF was frozen between passages. The AF was then used, either undiluted or diluted 1:1000 in phosphate-buffered saline (PBS) as noted in Table 1, to inoculate a new set of eggs for a second passage, and so on. A total of 69 passages were completed. Earlier passages were done at either about 34°C (passages 1-2) or about 30°C and on subsequent passages, the incubation temperature was shifted down either to about 28°C, or to about 26°C. In order to increase the possibility of the selection of the desired phenotype of a stable, attenuated virus, the initial serial passage was expanded to included five different limbs of the serial passage tree, A through E, as shown in Table 1.

TABLE 1: Passage history of the limbs A through E.

	Passage #				
Temperature	Limb A	Limb B	Limb C	Limb D	Limb E
34°C	1-2	1- 2	1-2	1-2	1-2
30°C	3-8	3-29	3-29	3-29	3-29
28°C		30-33*	30-68*	30-33	30-69
26°C	9-65	34-69*		34-65	

*= the infectious allantoic fluid was diluted 1:1000 in these passages

B. Virus isolates carried through the cold-adaptation procedure described in section A were tested for temperature sensitivity, i.e., a phenotype in which the cold-adapted virus grows at the lower, or permissive temperature (e.g., about 34°C), but no longer forms plaques

at a higher, or non-permissive temperature (e.g., about 37°C or about 39°C), as follows. At each cold-adaptation passage, the AF was titered by plaque assay at about 34°C. Periodically, individual plaques from the assay were clonally isolated by excision of the plaque area and placement of the excised agar plug in a 96-well tray containing a monolayer of MDCK cells. The 96-well trays were incubated overnight and the yield assayed for temperature sensitivity by CPE assay in duplicate 96-well trays incubated at about 34°C and at about 39°C. The percent of the clones that scored as temperature sensitive mutants by this assay, i.e., the number of viral plaques that grew at 34°C but did not grow at 39°C, divided by the total number of plaques, was calculated, and is shown in Table 2. Temperature sensitive isolates were then evaluated for protein synthesis at the non-permissive temperature by visualization of radiolabeled virus-synthesized proteins by SDS polyacrylamide gel electrophoresis (SDS-PAGE).

TABLE 2: Percent of isolated Clones that were temperature sensitive.

	Percent Temperature Sensitive				
Passage#	Limb A	Limb B	Limb C	Limb D	Limb E
p36	56%	66%	0%	66%	54%
p46		80%	60%		75%
p47			80%		
p48			100%		
p49		100%		100%	50%
p50			90%		
p51		100%			
p52					57%
p62	100%			100%	
p65			100%		
p66		100%			88%

From the clonal isolates tested for temperature sensitivity, two were selected for further study. Clone EIV-P821 was selected from the 49th passage of limb B and clone EIV-

P824 was selected from the 48th passage of limb C, as defined in Table 1. Both of these virus isolates were temperature sensitive, with plaque formation of both isolates inhibited at a temperature of about 39°C. At this temperature, protein synthesis was completely inhibited by EIV-P821, but EIV-P824 exhibited normal levels of protein synthesis. In addition, plaque formation by EIV-P821 was inhibited at a temperature of about 37°C, and at this temperature, late gene expression was inhibited, i.e., normal levels of NP protein were synthesized, reduced or no M1 or HA proteins were synthesized, and enhanced levels of the polymerase proteins were synthesized. The phenotype observed at 37°C, being typified by differential viral protein synthesis, was distinct from the protein synthesis phenotype seen at about 39°C, which was typified by the inhibition of synthesis of all viral proteins. Virus EIV-P821 has been deposited with the American Type Culture Collection (ATCC) under Accession No. ATCC VR-2625, and virus EIV-P824 has been deposited with the ATCC under Accession No. ATCC VR-2624.

C. Further characterization of the mutations in isolate EIV-P821 were carried out by reassortment analysis, as follows. Reassortment analysis in influenza viruses allows one skilled in the art, under certain circumstances, to correlate phenotypes of a given virus with putative mutations occurring on certain of the eight RNA segments that comprise an influenza A virus genome. This technique is described, for example, in Palese, et al., *ibid.* A mixed infection of EIV-P821 and an avian influenza virus, A/mallard/New York/6750/78 was performed as follows. MDCK cells were co-infected with EIV-P821 at a multiplicity of infection (MOI) of 2 pfu/cell and A/mallard/New York/6750/78 at an MOI of either 2, 5, or 10 pfu/cell. The infected cells were incubated at a temperature of about 34°C. The yields of the various co-infections were titered and individual plaques were isolated at about 34°C, and the resultant clonal isolates were characterized as to whether they were able to grow at about 39°C and about 37°C, and express their genes, i.e., synthesize viral proteins, at about 39°C, about 37°C, and about 34°C. Protein synthesis was evaluated by SDS-PAGE analysis of radiolabeled infected-cell lysates. The HA, NP and NS-1 proteins of the two parent

viruses, each of which is encoded by a separate genome segment, were distinguishable by SDS-PAGE analysis, since these particular viral proteins, as derived from either the equine or the avian influenza virus, migrate at different apparent molecular weights. In this way it was possible, at least for the HA, NP, and NS-1 genes, to evaluate whether certain phenotypes of the parent virus, e.g., the temperature sensitive and the protein synthesis phenotypes, co-segregate with the genome segments carrying these genes. The results of the reassortment analyses investigating co-segregation of a) the mutation inhibiting plaque formation, i.e., the induction of CPE, at a non-permissive temperature of about 39°C or b) the mutation inhibiting protein synthesis at a non-permissive temperature of about 39°C with each of the EIV-P821 HA, NP and NS-1 proteins are shown in Tables 3 and 4, respectively.

TABLE 3: Reassortment analysis of the EIV-P821 39°C plaque formation phenotype with avian influenza virus, A/mallard/New York/6750/78

Gene	Virus	ts+[1]	ts-[2]
HA	avian	26	13
	equine	11	44
NP	avian	37	8
	equine	0	49
NS-1	avian	9	8
	equine	12	20

[1] number of clonal isolates able to induce CPE in tissue culture cells at a temperature of about 39°C.
[2] number of clonal isolates inhibited in the ability to induce CPE in tissue culture cells at a temperature of about 39°C.

TABLE 4: Reassortment analysis of the EIV-P821 39°C protein synthesis phenotype with avian influenza virus, A/mallard/New York/6750/78

Gene	Virus	ts+[1]	ts-[2]
HA	avian	18	1
	equine	11	7
NP	avian	34	5
	equine	7	8
NS-1	avian	10	4
	equine	14	5

[1] number of clonal isolates which synthesize all viral proteins at a temperature of about 39°C.
[2] number of clonal isolates inhibited in the ability to synthesize all viral proteins at a temperature of about 39°C.

The results demonstrated an association of the equine NP gene with a mutation causing the inability of EIV-P821 to form plaques at a non-permissive temperature of about 39°C, but the results did not suggest an association of any of the HA, NP, or NS-1 genes with a mutation causing the inability of EIV-P821 to express viral proteins at a non-permissive temperature of about 39°C. Thus, these data also demonstrated that the plaque formation phenotype and the protein synthesis phenotype observed in virus EIV-P821 were the result of separate mutations.

D. Studies were also conducted to determine if cold-adapted equine influenza viruses of the present invention have a dominant interference phenotype, that is, whether they dominate in mixed infection with the wild type parental virus A/Kentucky/1/91 (H3N8). The dominant interference phenotype of viruses EIV-P821 and EIV-P824 were evaluated in the following manner. Separate monolayers of MDCK cells were singly infected with the parental virus A/Kentucky/1/91 (H3N8) at an MOI of 2, singly infected with either cold-adapted virus EIV-P821 or EIV-P824 at an MOI of 2, or simultaneously doubly infected with both the parental virus and one of the cold adapted viruses at an MOI of 2+2, all at a temperature of about 34°C. At 24 hours after infection, the media from the cultures were

harvested and the virus yields from the various infected cells were measured by duplicate plaque assays performed at temperatures of about 34 °C and about 39 °C. This assay took advantage of the fact that cold adapted equine influenza viruses EIV-P821 or EIV-P824 are temperature sensitive and are thus unable to form plaques at a non-permissive temperature of about 39°C, while the parental virus is able to form plaques at both temperatures, thus making it possible to measure the growth of the parental virus in the presence of the cold adapted virus. Specifically, the dominant interference effect of the cold adapted virus on the growth of the parental virus was quantitated by comparing the virus yield at about 39°C of the cells singly infected with parental virus to the yield of the parental virus in doubly infected cells. EIV-P821, in mixed infection, was able to reduce the yield of the parental virus by approximately 200 fold, while EIV-P824, in mixed infection, reduced the yield of the parental virus by approximately 3200 fold. This assay therefore showed that cold-adapted equine influenza viruses EIV-P821 and EIV-P824 both exhibit the dominant interference phenotype.

E. Virus isolate EIV-MSV+5 was derived from EIV-P821, as follows. EIV-P821 was passaged once in eggs, as described above, to produce a Master Seed Virus isolate, denoted herein as EIV-MSV0. EIV-MSV0 was then subjected to passage three additional times in eggs, the virus isolates at the end of each passage being designated EIV-MSV+1, EIV-MSV+2, and EIV-MSV+3, respectively. EIV-MSV+3 was then subjected to two additional passages in MDCK cells, as follows. MDCK cells were grown in 150 cm^2 tissue culture flasks in MEM tissue culture medium with Hanks Salts, containing 10% calf serum. The cells were then washed with sterile PBS and the growth medium was replaced with about 8 ml per flask of infection medium (tissue culture medium comprising MEM with Hanks Salts, 1 µg/ml TPCK trypsin solution, 0.125% bovine serum albumin (BSA), and 10 mM HEPES buffer). MDCK cells were inoculated with AF containing virus EIV-MSV+3 (for the first passage in MDCK cells) or virus stock harvested from EIV-MSV+4 (for the second passage in MDCK cells), and the viruses were allowed to adsorb for 1 hour at about

34°C. The inoculum was removed from the cell monolayers, the cells were washed again with PBS, and about 100 ml of infection medium was added per flask. The infected cells were incubated at about 34°C for 24 hours. The virus-infected MDCK cells were harvested by shaking the flasks vigorously to disrupt the cell monolayer, resulting in virus isolates EIV-MSV+4 (the first passage in MDCK cells), and EIV-MSV+5 (the second passage in MDCK cells).

Viruses EIV-MSV0 and EIV-MSV+5 were subjected to phenotypic analysis, as described in section B above, to determine their ability to form plaques and synthesize viral proteins at temperatures of about 34°C, about 37°C, and about 39°C. Both EIV-MSV0 and EIV-MSV+5 formed plaques in tissue culture cells at a temperature of about 34°C, and neither virus isolate formed plaques or exhibited detectable viral protein synthesis at a temperature of about 39°C. Virus EIV-MSV0 had a similar temperature sensitive phenotype as EIV-P821 at a temperature of about 37°C, i.e., it was inhibited in plaque formation, and late gene expression was inhibited. However, EIV-MSV+5, unlike its parent virus, EIV-P821, did form plaques in tissue culture at a temperature of about 37°C, and at this temperature, the virus synthesized normal amounts of all proteins. Virus EIV-MSV+5 has been deposited with the ATCC under Accession No. ATCC VR-2627.

Example 2

Therapeutic compositions of the present invention were produced as follows.

A. A large stock of EIV-P821 was propagated in eggs as follows. About 60 specific pathogen-free embryonated chicken eggs were candled and non-viable eggs were discarded. Stock virus was diluted to about 1.0×10^5 pfu/ml in sterile PBS. Virus was inoculated into the allantoic cavity of the eggs as described in Example 1A. After a 3-day incubation in a humidified chamber at a temperature of about 34°C, AF was harvested from the eggs according to the method described in Example 1A. The harvested AF was mixed with a stabilizer solution, for example A1/A2 stabilizer, available from Diamond Animal Health, Des Moines, IA, at 25 % V/V (stabilizer/AF). The harvested AF was batched in a centrifuge

tube and was clarified by centrifugation for 10 minutes at 1000 rpm in an IEC Centra-7R refrigerated table top centrifuge fitted with a swinging bucket rotor. The clarified fluid was distributed into 1-ml cryovials and was frozen at about -70°C. Virus stocks were titrated on MDCK cells by CPE and plaque assay at about 34°C.

B. A large stock of EIV-P821 was propagated in MDCK cells as follows. MDCK cells were grown in 150 cm^2 tissue culture flasks in MEM tissue culture medium with Hanks Salts, containing 10% calf serum. The cells were then washed with sterile PBS and the growth medium was replaced with about 8 ml per flask of infection medium. The MDCK cells were inoculated with virus stock at an MOI ranging from about 0.5 pfu per cell to about 0.005 pfu per cell, and the viruses were allowed to adsorb for 1 hour at about 34°C. The inoculum was removed from the cell monolayers, the cells were washed again with PBS, and about 100 ml of infection medium was added per flask. The infected cells were incubated at about 34°C for 24 hours. The virus-infected MDCK cells were harvested by shaking the flasks vigorously to disrupt the cell monolayer and stabilizer solution was added to the flasks at 25% V/V (stabilizer/virus solution). The supernatants were distributed aseptically into cryovials and frozen at -70°C.

C. Therapeutic compositions comprising certain cold-adapted temperature sensitive equine influenza viruses of the present invention were formulated as follows. Just prior to vaccination procedures, such as those described in Examples 3-7 below, stock vials of EIV-P821 or EIV-MSV +5 were thawed and were diluted in an excipient comprising either water, PBS, or in MEM tissue culture medium with Hanks Salts, containing 0.125% bovine serum albumin (BSA-MEM solution) to the desired dilution for administration to animals. The vaccine compositions were held on ice prior to vaccinations. All therapeutic compositions were titered on MDCK cells by standard methods just prior to vaccinations and wherever possible, an amount of the composition, treated identically to those administered to the animals, was titered after the vaccinations to ensure that the virus remained viable during the procedures.

Example 3

A therapeutic composition comprising cold-adapted equine influenza virus EIV-P821 was tested for safety and its ability to replicate in three horses showing detectable prior immunity to equine influenza virus as follows. EIV-P821, produced as described in Example 1A, was grown in eggs as described in Example 2A and was formulated into a therapeutic composition comprising 10^7 pfu EIV-P821/2ml BSA-MEM solution as described in Example 2C.

Three ponies having prior detectable hemagglutination inhibition (HAI) titers to equine influenza virus were inoculated with a therapeutic composition comprising EIV-P821 by the following method. Each pony was given a 2-ml dose of EIV-P821, administered intranasally using a syringe fitted with a blunt cannula long enough to reach past the false nostril, 1 ml per nostril.

The ponies were observed for approximately 30 minutes immediately following and at approximately four hours after vaccination for immediate type allergic reactions such as sneezing, salivation, labored or irregular breathing, shaking, anaphylaxis, or fever. The animals were further monitored on days 1-11 post-vaccination for delayed type allergic reactions, such as lethargy or anorexia. None of the three ponies in this study exhibited any allergic reactions from the vaccination.

The ponies were observed daily, at approximately the same time each day, starting two days before vaccination and continuing through day 11 following vaccination for clinical signs consistent with equine influenza. The ponies were observed for nasal discharge, ocular discharge, anorexia, disposition, heart rate, capillary refill time, respiratory rate, dyspnea, coughing, lung sounds, presence of toxic line on upper gum, and body temperature. In addition submandibular and parietal lymph nodes were palpated and any abnormalities were described. None of the three ponies in this study exhibited any abnormal reactions or overt clinical signs during the observation period.

To test for viral shedding in the animals, on days 0 through 11 following vaccination, nasopharyngeal swabs were collected from the ponies as described in Chambers, et al., 1995, *Equine Practice*, *17*, 19-23. Chambers, et al., *ibid.*. Briefly, two sterile Dacron polyester tipped applicators (available, e.g., from Hardwood Products Co., Guilford, ME) were inserted, together, into each nostril of the ponies. The swabs (four total, two for each nostril) were broken off into a 15-ml conical centrifuge tube containing 2.5 ml of chilled transport medium comprising 5% glycerol, penicillin, streptomycin, neomycin, and gentamycin in PBS at physiological pH. Keeping the samples on wet ice, the swabs were aseptically wrung out into the medium and the nasopharyngeal samples were divided into two aliquots. One aliquot was used to attempt isolation of EIV by inoculation of embryonated eggs, using the method described in Example 1. The AF of the inoculated eggs was then tested for its ability to cause hemagglutination, by standard methods, indicating the presence of equine influenza virus in the AF. On days 2 and 3 post-vaccination, the other aliquots were tested for virus by the Directigen® Flu A test, available from Becton-Dickinson (Cockeysville, MD).

Attempts to isolate EIV from the nasopharyngeal secretions of the three animals by egg inoculation were unsuccessful. However on days 2 and 3, all animals tested positive for the presence of virus shedding using the Directigen Flu A test, consistent with the hypothesis that EIV-P821 was replicating in the seropositive ponies.

To test the antibody titers to EIV in the inoculated animals described in this example, as well as in the animals described in Examples 4-7, blood was collected from the animals prior to vaccination and on designated days post-vaccination. Serum was isolated and was treated either with trypsin/periodate or kaolin to block the nonspecific inhibitors of hemagglutination present in normal sera. Serum samples were tested for hemagglutination inhibition (HAI) titers against a recent EIV isolate by standard methods, described, for example in the "Supplemental assay method for conducting the hemagglutination inhibition assay for equine influenza virus antibody" (SAM 124), provided by the U.S.D.A. National Veterinary Services Laboratory under 9 CFR 113.2.

The HAI titers of the three ponies are shown in Table 5. As can be seen, regardless of the initial titer, the serum HAI titers increased at least four-fold in all three animals after vaccination with EIV-P821.

These data demonstrate that cold-adapted equine influenza virus EIV-P821 is safe and non-reactogenic in sero-positive ponies, and that these animals exhibited an increase in antibody titer to equine influenza virus, even though they had prior demonstrable titers.

TABLE 5: HAI titers of vaccinated animals*

Animal	**HAI Titer (days after vaccination)**			
ID	**0**	**7**	**14**	**21**
18	40	80	160	160
19	10	20	40	80
25	20	40	320	80

* HAI titers are expressed as the reciprocal of the highest dilution of serum which inhibited hemagglutination of erythrocytes by a recent isolate of equine influenza virus.

Example 4

This Example discloses an animal study to evaluate the safety and efficacy of a therapeutic composition comprising cold-adapted equine influenza virus EIV-P821.

A therapeutic composition comprising cold-adapted equine influenza virus EIV-P821 was tested for attenuation, as well as its ability to protect horses from challenge with virulent equine influenza virus, as follows. EIV-P821, produced as described in Example 1, was grown in eggs as described in Example 2A and was formulated into a therapeutic composition comprising 10^7 pfu of virus/2ml water, as described in Example 2C. Eight EIV-seronegative ponies were used in this study. Three of the eight ponies were vaccinated with a 2-ml dose comprising 10^7 pfu of the EIV-P821 therapeutic composition, administered intranasally, using methods similar to those described in Example 3. One pony was given 10^7 pfu of the EIV-P821 therapeutic composition, administered orally, by injecting 6 ml of virus into the pharynx, using a 10-ml syringe which was adapted to create a fine spray by the following method. The protruding "seat" for the attachment of needles was sealed off using modeling clay and its cap was left in place. About 10 holes were punched through the

bottom of the syringe, i.e., surrounding the "seat," using a 25-gauge needle. The syringe was placed into the interdental space and the virus was forcefully injected into the back of the mouth. The remaining four ponies were held as non-vaccinated controls.

The vaccinated ponies were observed for approximately 30 minutes immediately following and at approximately four hours after vaccination for immediate type allergic reactions, and the animals were further monitored on days 1-11 post-vaccination for delayed type allergic reactions, both as described in Example 3. None of the four vaccinated ponies in this study exhibited any abnormal reactions from the vaccination.

The ponies were observed daily, at approximately the same time each day, starting two days before virus vaccination and continuing through day 11 following vaccination for clinical signs, such as those described in Example 3. None of the four vaccinated ponies in this study exhibited any clinical signs during the observation period. This result demonstrated that cold-adapted equine influenza virus EIV-P821 exhibits the phenotype of attenuation.

To test for viral shedding in the vaccinated animals, on days 0 through 11 following vaccination, nasopharyngeal swabs were collected from the ponies as described in Example 3. The nasopharyngeal samples were tested for virus in embryonated chicken eggs according to the method described in Example 3.

As shown in Table 6, virus was isolated from only one vaccinated animal using the egg method. However, as noted in Example 3, the lack of isolation by this method does not preclude the fact that virus replication is taking place, since replication may be detected by more sensitive methods, e.g., the Directigen Flu A test.

TABLE 6: Virus isolation in eggs after vaccination.

Animal ID	Virus Isolation (days after vaccination)											
	Route	0	1	2	3	4	5	6	7	8	9	10	11
91	IN	-	-	+	+	+	+	+	+	+	+	+	-
666	IN	-	-	-	-	-	-	-	-	-	-	-	-
673	IN	-	-	-	-	-	-	-	-	-	-	-	-
674	Oral	-	-	-	-	-	-	-	-	-	-	-	-

To test the antibody titers to equine influenza virus in the vaccinated animals, blood was collected from the animals prior to vaccination and on days 7, 14, 21, and 28 post-vaccination. Serum samples were isolated and were tested for hemagglutination inhibition (HAI) titers against a recent EIV isolate according to the methods described in Example 3.

The HAI titers of the four vaccinated ponies are shown in Table 7.

TABLE 7: HAI titers after vaccination.

Animal ID	HAI Titer (days after vaccination)					
	Route	0	7	14	21	28
91	IN	<10	<10	<10	<10	<10
666	IN	10	10	10	20	20
673	IN	10	10	10	20	20
674	Oral	20	40	40	40	40

Unlike the increase in HAI titer observed with the three animals described in the study in Example 3, the animals in this study did not exhibit a significant increase, i.e., greater than four-fold, in HAI titer following vaccination with EIV-P821.

Approximately four and one-half months after vaccine virus administration, all 8 ponies, i.e., the four that were vaccinated and the four non-vaccinated controls, were challenged by the following method. For each animal, 10^7 pfu of the virulent equine influenza virus strain A/equine/Kentucky/1/91 (H3N8) was suspended in 5 ml of water. A mask was connected to a nebulizer, and the mask was placed over the animal's muzzle, including the nostrils. Five (5) ml was nebulized for each animal, using settings such that it took 5-10 minutes to deliver the full 5 ml. Clinical observations, as described in Example 3,

were performed on all animals three days before challenge and daily for 11 days after challenge.

Despite the fact that the vaccinated animals did not exhibit marked increases in their HAI titers to equine influenza virus, all four vaccinated animals were protected against equine influenza virus challenge. None of the vaccinated animals showed overt clinical signs or fever, although one of the animals had a minor wheeze for two days. On the other hand, all four non-vaccinated ponies shed virus and developed clinical signs and fever typical of equine influenza virus infection. Thus, this example demonstrates that a therapeutic composition of the present invention can protect horses from equine influenza disease.

Example 5

This Example discloses an additional animal study to evaluate attenuation of a therapeutic composition comprising cold-adapted equine influenza virus EIV-P821, and its ability to protect vaccinated horses from subsequent challenge with virulent equine influenza virus. Furthermore, this study evaluated the effect of exercise stress on the safety and efficacy of the therapeutic composition.

A therapeutic composition comprising cold-adapted equine influenza virus EIV-P821 was tested for safety and efficacy in horses, as follows. EIV-P821, produced as described in Example 1, was grown in eggs as described in Example 2A and was formulated into a therapeutic composition comprising 10^7 pfu virus/5ml water, as described in Example 2C. Fifteen ponies were used in this study. The ponies were randomly assigned to three groups of five animals each, as shown in Table 8, there being two vaccinated groups and one unvaccinated control group. The ponies in group 2 were exercise stressed before vaccination, while the ponies in vaccinate group 1 were held in a stall.

TABLE 8: Vaccination/challenge protocol.

Group	No. Ponies	Exercise	Vaccine	Challenge
1	5	-	Day 0	Day 90
2	5	Days -4 to 0	Day 0	Day 90
3	5	-	-	Day 90

The ponies in group 2 were subjected to exercise stress on a treadmill prior to vaccination, as follows. The ponies were acclimated to the use of the treadmill by 6 hours of treadmill use at a walk only. The actual exercise stress involved a daily exercise regimen starting 4 days before and ending on the day of vaccination (immediately prior to vaccination). The treadmill exercise regimen is shown in Table 9.

TABLE 9: Exercise regimen for the ponies in Group 2.

Speed (m/sec)	Time (min.)	Incline (°)
1.5	2	0
3.5	2	0
3.5	2	7
4.5 †	2	7
5.5 †	2	7
6.5 †	2	7
7.5 †	2	7
8.5 †	2	7
3.5	2	7
1.5	10	0†

† Speed, in meters per second (m/sec) was increased for each animal every 2 minutes until the heart rate reached and maintained ≥200 beats per minute

Groups 1 and 2 were given a therapeutic composition comprising 10^7 pfu of EIV-P821, by the nebulization method described for the challenge described in Example 4. None of the vaccinated ponies in this study exhibited any immediate or delayed allergic reactions from the vaccination.

The ponies were observed daily, at approximately the same time each day, starting two days before vaccination and continuing through day 11 following vaccination for clinical

signs, such as those described in Example 3. None of the vaccinated ponies in this study exhibited any overt clinical signs during the observation period.

To test for viral shedding in the vaccinated animals, before vaccination and on days 1 through 11 following vaccination, nasopharyngeal swabs were collected from the ponies as described in Example 3. The nasopharyngeal samples were tested for virus in embryonated chicken eggs according to the method described in Example 3. Virus was isolated from the vaccinated animals, i.e., Groups 1 and 2, as shown in Table 10.

TABLE 10: Virus isolation after vaccination.

Group	Animal ID	Exercise	Virus Isolation (days after vaccination)											
			0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1	12	No	-	-	+	+	+	+	+	-	+	+	-	-
	16		-	-	+	+	+	+	+	-	-	-	-	-
	17		-	-	+	+	+	+	+	+	+	-	+	-
	165		-	-	-	-	-	-	-	-	-	-	-	-
	688		-	-	-	-	-	+	-	+	-	-	-	-
2	7	Yes	-	-	-	+	+	+	+	-	-	-	-	-
	44		-	-	-	-	-	-	-	-	-	-	-	-
	435		-	-	+	+	+	+	-	-	-	-	-	-
	907		-	-	-	+	-	+	+	-	-	-	-	-
	968		-	-	-	-	-	+	-	+	-	-	-	-

To test the antibody titers to equine influenza virus in the vaccinated animals, blood was collected prior to vaccination and on days 7, 14, 21, and 28 post-vaccination. Serum samples were isolated and were tested for HAI titers against a recent EIV isolate according to the methods described in Example 3. These titers are shown in Table 11.

TABLE 11: HAI titers after vaccination and after challenge on day 90.

Group	Animal ID	Day Post-vaccination									
		-1	7	14	21	28	91	105	112	119	126
1	12	<10	<10	<10	<10	<10	<10	80	320	320	640
1	16	<10	<10	20	20	<10	<10	20	160	320	320
1	17	<10	<10	10	10	10	10	80	160	160	160
1	165	<10	<10	10	10	10	10	80	80	80	80
1	688	<10	<10	20	20	20	20	20	20	20	40
2	7	<10	<10	10	10	<10	<10	20	80	80	40
2	44	<10	<10	20	20	20	10	80	320	320	320
2	435	<10	<10	20	20	10	<10	20	80	80	80
2	907	<10	<10	10	10	20	10	10	40	80	80
2	968	<10	<10	<10	<10	<10	<10	40	160	160	160
3	2						<10	80	640	640	320
3	56						<10	80	320	320	320
3	196						<10	20	160	80	80
3	198						10	40	160	320	320
3	200						<10	20	80	80	40

Group	Description
1	Vaccination only
2	Vaccination and Exercise
3	Control

On day 90 post vaccination, all 15 ponies were challenged with 10^7 pfu of equine influenza virus strain A/equine/Kentucky/1/91 (H3N8) by the nebulizer method as described in Example 4. Clinical observations, as described in Example 3, were performed on all animals three days before challenge and daily for 11 days after challenge. There were no overt clinical signs observed in any of the vaccinated ponies. Four of the five non-vaccinated ponies developed fever and clinical signs typical of equine influenza virus infection.

Thus, this example demonstrates that a therapeutic composition of the present invention protects horses against equine influenza disease, even if the animals are stressed prior to vaccination.

Example 6

This Example compared the infectivities of therapeutic compositions of the present invention grown in eggs and grown in tissue culture cells. From a production standpoint, there is an advantage to growing therapeutic compositions of the present invention in tissue

culture rather than in embryonated chicken eggs. Equine influenza virus, however, does not grow to as high a titer in cells as in eggs. In addition, the hemagglutinin of the virus requires an extracellular proteolytic cleavage by trypsin-like proteases for infectivity. Since serum contains trypsin inhibitors, virus grown in cell culture must be propagated in serum-free medium that contains trypsin in order to be infectious. It is well known by those skilled in the art that such conditions are less than optimal for the viability of tissue culture cells. In addition, these growth conditions may select for virus with altered binding affinity for equine cells, which may affect viral infectivity since the virus needs to bind efficiently to the animal's nasal mucosa to replicate and to stimulate immunity. Thus, the objective of the study disclosed in this example was to evaluate whether the infectivity of therapeutic compositions of the present invention was adversely affected by growth for multiple passages in *in vitro* tissue culture.

EIV-P821, produced as described in Example 1, was grown in eggs as described in Example 2A or in MDCK cells as described in Example 2B. In each instance, the virus was passaged five times. EIV-P821 was tested for its cold-adaptation and temperature sensitive phenotypes after each passage. The egg and cell-passaged virus preparations were formulated into therapeutic compositions comprising 10^7 pfu virus/2ml BSA-MEM solution, as described in Example 2C, resulting in an egg-grown EIV-P821 therapeutic composition and an MDCK cell-grown EIV-P821 therapeutic composition, respectively.

Eight ponies were used in this study. Serum from each of the animals was tested for HAI titers to equine influenza virus prior to the study. The animals were randomly assigned into one of two groups of four ponies each. Group A received the egg-grown EIV-P821 therapeutic composition, and Group B received the MDCK-grown EIV-P821 therapeutic composition, prepared as described in Example 2B. The therapeutic compositions were administered intranasally by the method described in Example 3.

The ponies were observed daily, at approximately the same time each day, starting two days before vaccination and continuing through day 11 following vaccination for allergic

reactions or clinical signs as described in Example 3. No allergic reactions or overt clinical signs were observed in any of the animals.

Nasopharyngeal swabs were collected before vaccination and daily for 11 days after vaccination. The presence of virus material in the nasal swabs was determined by the detection of CPE on MDCK cells infected as described in Example 1, or by inoculation into eggs and examination of the ability of the infected AF to cause hemagglutination, as described in Example 3. The material was tested for the presence of virus only, and not for titer of virus in the sample. Virus isolation results are listed in Table 12. Blood was collected and serum samples from days 0, 7, 14, 21 and 28 after vaccination were tested for hemagglutination inhibition antibody titer against a recent isolate. HAI titers are also listed in Table 12.

TABLE 12: HAI titers and virus isolation after vaccination.

Group[2]	ID	HAI Titer (DPV[3])					Virus Isolation [1] (DPV[3])											
		0	7	14	21	28	0	1	2	3	4	5	6	7	8	9	10	11
1	31	<10	20	160	160	160	-	EC	-	C	EC	EC	C	C	EC	-	-	-
	37	<10	40	160	160	160	-	EC	C	C	EC	C	C	C	-	-	-	-
	40	<10	20	80	160	80	-	EC	EC	C	-	C	EC	C	-	EC	EC	-
	41	<10	40	160	160	80	-	EC	EC	C	EC	C	EC	EC	-	-	-	-
2	32	<10	<10	80	80	40	-	EC	-	C	-	C	-	C	-	EC	-	-
	34	<10	20	160	160	160	-	EC	-	C	EC	C	EC	C	-	-	-	-
	35	<10	<10	80	80	40	-	EC	-	C	-	C	-	C	-	EC	-	-
	42	<10	<10	80	80	40	-	-	-	C	-	C	EC	EC	-	-	-	-

[1] E = Egg isolation positive; C=CPE isolation positive; - = virus not detected by either of the methods
[2] Group 1: Virus passaged 5X in MDCK cells; Group 2: Virus passaged 5X in Eggs
[3] Days Post-vaccination

The results in Table 12 show that there were no significant differences in infectivity or immunogenicity between the egg-grown and MDCK-grown EIV-P821 therapeutic compositions.

Example 7

This example evaluated the minimum dose of a therapeutic composition comprising a cold-adapted equine influenza virus required to protect a horse from equine influenza virus infection.

The animal studies disclosed in Examples 3-6 indicated that a therapeutic composition of the present invention was efficacious and safe. In those studies, a dose of 10^7 pfu, which correlates to approximately 10^8 $TCID_{50}$ units, was used. However, from the standpoints of cost and safety, it is advantageous to use the minimum virus titer that will protect a horse from disease caused by equine influenza virus. In this study, ponies were vaccinated with four different doses of a therapeutic composition comprising a cold-adapted equine influenza virus to determine the minimum dose which protects a horse against virulent equine influenza virus challenge.

EIV-P821, produced as described in Example 1A, was passaged and grown in MDCK cells as described in Example 2B and was formulated into a therapeutic composition comprising either 2 x 10^4, 2 x 10^5, 2 x 10^6, or 2 x 10^7 $TCID_{50}$ units/1 ml BSA-MEM solution as described in Example 2C. Nineteen horses of various ages and breeds were used for this study. The horses were assigned to four vaccine groups, one group of three horses and three groups of four horses, and one control group of four horses (see Table 13). Each of the ponies in the vaccine groups were given a 1-ml dose of the indicated therapeutic composition, administered intranasally by methods similar to those described in Example 3.

TABLE 13: Vaccination protocol.

Group No.	No. Animals	Vaccine Dose, $TCID_{50}$ Units
1	3	2 x 10^7
2	4	2 x 10^6
3	4	2 x 10^5
4	4	2 x 10^4
5	4	control

The ponies were observed for approximately 30 minutes immediately following and at approximately four hours after vaccination for immediate type reactions, and the animals were further monitored on days 1-11 post-vaccination for delayed type reactions, both as described in Example 3. None of the vaccinated ponies in this study exhibited any abnormal reactions or overt clinical signs from the vaccination.

Blood for serum analysis was collected 3 days before vaccination, on days 7, 14, 21, and 28 after vaccination, and after challenge on Days 35 and 42. Serum samples were tested for HAI titers against a recent EIV isolate according to the methods described in Example 3. These titers are shown in Table 14. Prior to challenge on day 29, 2 of the 3 animals in group 1, 4 of the 4 animals in group 2, 3 of the 4 animals in group 3, and 2 of the 4 animals in group 4 showed at least 4-fold increases in HAI titers after vaccination. In addition, 2 of the 4 control horses also exhibited increases in HAI titers. One interpretation for this result is that the control horses were exposed to vaccine virus transmitted from the vaccinated horses, since all the horses in this study were housed in the same barn.

TABLE 14: · HAI titers post-vaccination and post-challenge, and challenge results.

No.	Dose in $TCID_{50}$ units	Animal ID	Vaccination on Day 0, Challenge on Day 29							Chall. Sick
			-1	7	14	21	28	35	42	+/-
1	$2x10^7$	41	<10	<10	10	40	10	20	80	-
		42	40	40	40	40	40	<10	80	-
		200	<10	<10	80	40	160	40	40	-
2	$2x10^6$	679	<10	10	40	40	40	20	20	-
		682	<10	<10	40	40	40	40	40	-
		795	20	80	160	160	320	320	640	-
		R	<10	10	40	20	160	40	40	-
3	$2x10^5$	73	<10	<10	160	40	80	160	160	-
		712	<10	<10	20	20	40	40	20	-
		720	<10	20	80	40	80	80	160	-
		796	<10	<10	<10	<10	<10	10	80	+
4	$2x10^4$	75	<10	<10	<10	<10	<10	<10	160	+
		724	<10	>10	<10	<10	<10	20	320	+
		789	<10	10	320	160	320	320	320	-
		790	<10	<10	80	40	160	80	40	
5	Control	12	<10	<10	<10	20	20	40	40	-
		22	10	20	40	10	160	40	640	-
		71	<10	<10	<10	<10	10	20	160	+
		74	<10	<10	<10	<10	<10	<10	20	+

On day 29 post vaccination, all 19 ponies were challenged with equine influenza virus strain A/equine/Kentucky/1/91 (H3N8) by the nebulizer method as described in Example 4. The challenge dose was prospectively calculated to contain about 10^8 $TCID_{50}$ units of challenge virus in a volume of 5 ml for each animal. Clinical observations, as described in Example 3, were monitored beginning two days before challenge, the day of challenge, and for 11 days following challenge. As shown in Table 14, no animals in groups 1 or 2 exhibited clinical signs indicative of equine influenza disease, and only one out of four animals in group 3 became sick. Two out of four animals in group 4 became sick, and only two of the four control animals became sick. The results in Table 14 suggest a correlation between seroconversion and protection from disease, since, for example, the two control

animals showing increased HAI titers during the vaccination period did not show clinical signs of equine influenza disease following challenge. Another interpretation, however, was that the actual titer of the challenge virus may have been less than the calculated amount of 10^8 $TCID_{50}$ units, since, based on prior results, this level of challenge should have caused disease in all the control animals.

Nonetheless, the levels of seroconversion and the lack of clinical signs in the groups that received a therapeutic composition comprising at least 2 x 10^6 $TCID_{50}$ units of a cold-adapted equine influenza virus suggests that this amount was sufficient to protect a horse against equine influenza disease. Furthermore, a dose of $2x10^5$ $TCID_{50}$ units induced seroconversion and gave clinical protection from challenge in 3 out of 4 horses, and thus even this amount may be sufficient to confer significant protection in horses against equine influenza disease.

Example 8

This example discloses an animal study to evaluate the duration of immunity of a therapeutic composition comprising cold-adapted equine influenza virus EIV-P821.

A therapeutic composition comprising cold-adapted equine influenza virus EIV-P821, produced as described in Example 1, was grown in eggs similarly to the procedure described in Example 2A, was expanded by passage in MDCK cells similarly to the procedure described in Example 2B, and was formulated into a therapeutic composition as described in Example 2C. Thirty horses approximately 11 to 12 months of age were used for this study. Nineteen of the horses were each vaccinated intranasally into one nostril using a syringe with a delivery device tip attached to the end, with a 1.0 ml dose comprising 6 logs of $TCID_{50}$ units of the EIV-P821 therapeutic composition. Vaccinations were performed on Day 0.

The horses were observed on Day 0 (before vaccination and up to 4 hours post-vaccination) and on Study Days 1, 2, 3, 7, 15, and 169 post-vaccination. On these days, a distant examination for a period of at least 15 minutes was performed. This distant examination included observation for demeanor, behavior, coughing, sneezing, and nasal

discharge. The examination on Day 169 also served to confirm that the horses were in a condition of health suitable for transport to the challenge site which was located approximately 360 miles from the vaccination site.

The animals were acclimated to the challenge site and were observed approximately daily by a veterinarian or animal technician for evidence of disease. A general physical examination was performed on Day 171 post-vaccination to monitor the following: demeanor, behavior, coughing, sneezing, and nasal discharge. From Days 172 to 177, similar observations as well as rectal temperature were recorded, according to the judgment of the attending veterinarian for any individual horse with abnormal clinical presentation.

No vaccinated horses showed any adverse reactions post-vaccination. One vaccinate was found dead about two months after vaccination. This horse showed no evidence of adverse reaction when observed for at least one month after vaccination. Although no cause of death could be firmly established, the death was not instantaneous and was considered to be consistent with possible contributing factors such as colic, bone fracture, or severe worm burden. Since there was no other evidence for any adverse reactions post-vaccination in any other vaccinates, it is highly unlikely that the vaccine contributed to any adverse reaction in this case.

Challenges were performed on Day 181 post-vaccination. The following wild-type isolate of equine influenza virus previously shown to cause disease in horses was used as the challenge virus: A/equine/2/Kentucky/91. Prior to infection of each challenge group, the challenge material was rapidly thawed at approximately 37°C. The virus was diluted with phosphate-buffered saline to a total volume of approximately 21 ml. The diluted material was stored chilled on ice until immediately before inoculation. Before inoculation and at the end of nebulization for each challenge group, a sample of diluted challenge virus was collected for pre-and post-inoculation virus titer confirmation. Vaccinates and controls were randomly assigned to 4 challenge groups of 6 horses each and one challenge group of 5

horses so that each challenge group contained a mixture of 4 vaccinates and 2 controls or 3 vaccinates and 2 controls.

Challenge virus in aerosol form was delivered through a tube inserted through a small opening centrally in the plastic ceiling with an ultrasonic nebulizer (*e.g.*, DeVilbiss Model 099HD, DeVilbiss Healthcare Inc., Somerset, Pennsylvania) for a period of approximately 10 minutes. The horses remained in the chamber for a further period of approximately 30 minutes after the nebulization had been completed (total exposure time, approximately 40 minutes). At that time, the plastic was removed to vent the chamber, and the horses were released and returned to their pen. The challenge procedure was repeated for each group.

All statistical methods in this study were performed using SAS (SAS Institute, Cary, NC), and $P < 0.05$ was considered to be statistically significant. Beginning on Day 178 post-vaccination (three days prior to challenge) through Day 191 (day 10 post-challenge), the horses were observed daily by both distant and individual examinations. Rectal temperatures were measured at these times. Data from day 0 (challenge day) to day 10 were included in the analysis; see Table 15.

TABLE 15: Effect of challenge on daily temperatures (°C) in vaccinated and control horses (least squares means).

Day post challenge	Vaccinated (n=19)	non-vaccinated (n=10)	P-value
0	100.7	100.8	0.8434
1	100.5	100.4	0.7934
2	103.4	104.9	0.0024
3	101.8	103.9	0.0001
4	101.5	103.2	0.0002
5	101.7	103.8	0.0001
6	101.3	103.6	0.0001
7	100.7	102.3	0.0007
8	100.5	101.4	0.0379
9	100.1	100.3	0.7416
10	100.3	100.5	0.7416
pooled SEM*	0.27	0.38	

*Standard error of the mean

Table 15 shows that on days 2 through 8, vaccinated horses had lower temperatures ($P < 0.05$) than the non-vaccinated control horses.

The distant examination consisted of a period of 20 minutes where the following observations were made: coughing, nasal discharge, respiration, and depression. Scoring criteria are shown in Table 16.

TABLE 16: Clinical signs and scoring index.

Clinical Sign	Description	Score
Coughing	normal during observation period of 15 min	0
	coughing once during observation	1
	coughing twice or more during observation	2
Nasal discharge	normal	0
	abnormal, serous	1
	abnormal, mucopurulent	2
	abnormal, profuse	3
Respiration	normal	0
	abnormal (dyspnea, tachypnea)	1
Depression	normal	0
	depression present†	1

†Depression was assessed by subjective evaluation of individual animal behavior that included the following: failure to approach food rapidly, general lethargy, inappetence, and anorexia.

Each horse was scored for each of these categories. Additionally, submandibular lymph nodes were palpated to monitor for possible bacterial infection. In any case where there was a different value recorded for a subjective clinical sign score from an observation on the same day at the distant versus the individual examination, the greater score was used in the compilation and analysis of results. For purposes of assessing the health of the horses prior to final disposition, distant examinations were performed at 14, 18, and 21 days post-challenge. Data from days 1 through 10 post-challenge were included in the analysis. These scores were summed on each day for each horse, and the vaccinates and controls were compared using the Wilcoxon rank sums test. In addition, these scores were summed across all days for each horse, and compared in the same manner. The mean ranks and mean clinical scores are shown in Tables 17 and 18, respectively. Five days post-challenge, the mean rank of scores in the vaccinated horses was lower ($P < 0.05$) than in the non-vaccinated control horses; and this effect continued on days 6, 7, 8, 9, and 10 ($P < 0.05$). The

cumulative rank over the entire test period was also lower ($P < 0.05$) in the vaccinated horses than the non-vaccinated controls.

TABLE 17: Effect of challenge on clinical sign scores in vaccinated and control horses (mean rank).

Day post challenge	Vaccinated (n=19), mean rank*	Non-vaccinated (n=10), mean rank	P-value
0	13.6	17.6	0.1853
1	16.4	12.4	0.2015
2	15.1	14.9	0.9812
3	13.3	18.3	0.1331
4	13.5	17.9	0.1721
5	12.4	19.9	0.0237
6	12.7	19.4	0.0425
7	12.1	20.6	0.0074
8	12.6	19.6	0.0312
9	13.1	18.7	0.0729
10	12.3	20.1	0.0135
total over 11 days	11.8	21.2	0.0051

*By Wilcoxon rank sum test.

TABLE 18: Effect of challenge on clinical sign scores in vaccinated and control horses (mean scores).

Day post challenge	Vaccinated (n=19)	Non-vaccinated (n=10)
0	1.2	1.6
1	1.5	0.9
2	2.4	2.5
3	3.2	4.1
4	3.4	4.3
5	3.2	4.7
6	3.4	4.8
7	3.3	4.7
8	3.2	4.5
9	3.2	3.9
10	2.4	3.4

Nasopharyngeal swabs were obtained on the day prior to challenge and on days 1 to 8 post-challenge, as described in Example 3, and tested for shed virus by cell culture assay. The percent of horses shedding challenge virus in each group is shown in Table 19. The percent of horses shedding the challenge virus in the vaccinated group was lower ($P < 0.05$)

on days 5 and 6 post-challenge than in the non-vaccinated controls. The mean number of days the challenge virus was shed was also lower ($P < 0.05$) in the vaccinated group as compared to the non-vaccinated controls.

TABLE 19: Percent of horses shedding virus per day post-challenge and mean number of days of shedding per group.

Day post challenge	Vaccinated (n=19)	Non-vaccinated (n=10)
-1	0	0
1	63.2	90
2	100	100
3	84.2	100
4	100	100
5	47.4	88.9*
6	10.5	77.8*
7	5.3	20
8	0	0
average number of days shedding	4.1	5.6*

*Within a time point, vaccinates different from non-vaccinates ($P < 0.05$) by either Fisher's exact test (percent data) or Wilcoxon rank sums test (days shedding).

The scores from clinical signs relevant to influenza and the objective temperature measurements both demonstrated a statistically significant reduction in the group of vaccinates when compared to those from the control group; this is consistent with an interpretation that the vaccine conferred significant protection from disease.

The ability of horses to shed influenza virus post-challenge was also significantly reduced in vaccinates as compared to controls in both the incidence of horses positive for shedding on certain days post-challenge and the mean number of days of shedding per horse. This decreased shedding by vaccinates is important in that it should serve to reduce the potential for exposure of susceptible animals to the wild-type virus in an outbreak of influenza.

The results of this study are consistent with the interpretation that the vaccine safely conferred protection for 6 months from clinical disease caused by equine influenza and reduced the potential for the spread of naturally occurring virulent equine influenza virus.

While the degree of protection from disease was not complete (13 out of 19 vaccinates were protected, while 10/10 controls were sick), there was a clear reduction in the severity and duration of clinical illness and a noticeable effect on the potential for viral shedding after exposure to a virulent strain of equine influenza. The finding that both vaccinates and controls were seronegative immediately prior to challenge at 6 months post-immunization suggests that immunity mediated by something other than serum antibody may be of primary importance in the ability of this vaccine to confer measurable and durable protection.

Example 9

This Example discloses an animal study to evaluate the ability of a therapeutic composition comprising cold-adapted equine influenza virus EIV-P821 to aid in the prevention of disease following exposure to a heterologous strain of equine influenza virus.

The heterologous strain tested was A/equine/2/Saskatoon/90, described genetically as a Eurasian strain (obtained from Hugh Townsend, University of Saskatchewan). Twenty female Percheron horses approximately 15 months of age (at the time of vaccination) were used for the efficacy study. The horses were assigned to two groups, one group of 10 to be vaccinated and another group of 10 to serve as non-vaccinated controls. On day 0, the vaccinate group was vaccinated in the manner described in Example 8.

The challenge material, *i.e.* equine flu strain A/equine/2/Saskatoon/90 [H3N8] was prepared similarly to the preparation in Example 8. Vaccinates and controls were randomly assigned to 4 challenge groups of 5 horses each such that each challenge group contained a mixture of 2 vaccinates and three controls or vice versa. The challenge procedure was similar to that described in Example 8. Challenges were performed on Day 28 post-vaccination.

Clinical observations were performed for the vaccinates and controls on Day -4 and on Study Days 0 (before vaccination and up to 4 hours post-vaccination), 1 to 7, 12, 15 to 17, 19 to 23, 25 to 38, and 42. For days on which clinical observations were performed during Days -4 to 42, clinical observations including rectal temperature were recorded according to

the judgment of the attending veterinarian for any individual horse with abnormal clinical presentation. Horses were scored using the same criteria as in Example 8 (Table 15). Distant examinations were performed on these days as described in Example 8. On Day 20 and from Days 25 to 38, the horses were also observed by both distant and individual examinations (also performed as described in Example 8).

Rectal temperatures were measured daily beginning 3 days prior to challenge, and continuing until 10 days post-challenge. Day 0 is the day relative to challenge. Data from days 0 through 10 were included in the analysis. Statistical methods and criteria were identical to those used in Example 8. On days 2, 5 and 7, vaccinated horses had statistically significant lower body temperatures than the non-vaccinated control horses (Table 20).

TABLE 20: Effect of challenge on daily temperatures (°C) in vaccinated and control horses (least squares means).

Day post challenge	Vaccinated (n=10)	Non-vaccinated (n=10)	P-value
0	99.9	99.8	0.9098
1	100.5	100.3	0.4282
2	101.0	102.8	0.0001
3	100.7	100.6	0.7554
4	101.0	101.3	0.4119
5	100.8	102.1	0.0004
6	100.4	100.4	0.9774
7	100.3	101.1	0.0325
8	100.6	100.7	0.8651
9	100.5	100.6	0.8874
10	100.5	100.1	0.2465

Standard error of the mean = 0.249.

Data from days 1 through 10 post-challenge were included in the analysis. These scores were summed on each day for each horse, and the vaccinates and controls were compared using the Wilcoxon rank sums test. All statistical methods were performed as described in Example 9. In addition, these scores were summed across all days for each horse, and compared in the same manner. Mean ranks are shown in Table 21.

TABLE 21: Effect of challenge on clinical sign scores in vaccinated and control horses (mean rank).

Day post challenge	Vaccinated (n=10)	Non-vaccinated (n=10)	P-value*
1	8.85	12.15	0.1741
2	8.80	12.20	0.1932
3	8.90	12.10	0.2027
4	7.60	13.40	0.0225
5	6.90	14.10	0.0053
6	7.00	14.00	0.0059
7	6.90	14.10	0.0053
8	7.60	13.40	0.0251
9	6.90	14.10	0.0048
10	6.10	14.90	0.0006
total over 10 days	5.70	15.30	0.0003

*By Wilcoxon 2 sample test.

On day 4 post-challenge, the mean rank of scores in the vaccinated horses was lower ($P < 0.05$) than the non-vaccinated control horses, and this effect continued throughout the remainder of the study ($P < 0.05$). The cumulative rank over the entire test period was also lower in the vaccinated horses than the non-vaccinated controls ($P < 0.05$).

Nasopharyngeal swabs were collected on days 1 and 8 post-challenge, as described in Example 3. The nasal samples were analyzed for the presence of virus by cell inoculation with virus detection by cytopathogenic effect (CPE) or by egg inoculation with virus detection by hemagglutination (HA). The cell-culture assay was performed as generally described by Youngner *et al.*, 1994, *J. Clin. Microbiol.* 32, 750-754. Serially diluted nasal samples were added to wells containing monolayers of Madin Darby Canine Kidney (MDCK) cells. After incubation, wells were examined for the presence and degree of cytopathogenic effect. The quantity of virus in $TCID_{50}$ units was calculated by the Reed-Muench technique. The egg infectivity assay was performed as described in Example 1. The percent of horses shedding challenge virus for each assay in each group is shown in Tables 22 and 23. The percent of horses shedding the challenge virus in the vaccinated group was lower ($P < 0.05$) on days 2 through 7 post-challenge by either method. No differences were seen on days 1 or 8 post-challenge. The number of days the challenge virus was shed was

also lower ($P < 0.05$) in the vaccinated group as compared to the non-vaccinated controls; see Tables 22 and 23.

TABLE 22: Percent of horses shedding virus following challenge - cell culture assay.

Day post challenge	Vaccinated (n=10)	Non-vaccinated (n=10)
1	0	0
2	0	70*
3	0	70*
4	20	100*
5	10	100*
6	20	100*
7	0	80*
8	0	30
average number of days shedding	0.5	5.5*

*Within a time point, vaccinates different from non-vaccinates, $P < 0.05$ by either Fisher's exact test (percent data) or Wilcoxon 2 sample test (days shedding)

TABLE 23: Percent of horses shedding virus following challenge - egg infectivity assay.

Day post challenge	Vaccinated (n=10)	Non-vaccinated (n=10)
1	0	0
2	0	70*
3	10	70*
4	0	90*
5	10	70*
6	20	90*
7	0	50*
8	0	0
average number of days shedding	0.4	4.4*

*Within a time point, vaccinates different from non-vaccinates, $P < 0.05$ by either Fisher's exact test (percent data) or Wilcoxon 2 sample test (days shedding).

The extent (severity and duration) of clinical signs of influenza among vaccinates was substantially reduced relative to the controls. The scores from clinical signs relevant to influenza and the objective temperature measurements both demonstrated a statistically significant reduction in the group of vaccinates when compared to those from the control

group; indicating that the vaccine conferred significant protection from disease by the heterologous strain.

The ability of horses to shed influenza virus post-challenge was also significantly reduced in vaccinates as opposed to controls in both the incidence of horses positive for shedding on certain days post-challenge and the mean number of days of shedding per horse. This decreased shedding by vaccinates is important in that it should serve to reduce the potential for exposure of susceptible animals to the wild-type virus in an outbreak of influenza.

Overall, the results of this study show that the vaccine conferred protection against a heterologous challenge by a member of the Eurasian lineage of equine influenza virus strains.

Example 10

This Example discloses an animal study to evaluate the ability of a therapeutic composition comprising cold-adapted equine influenza virus EIV-P821 to aid in the prevention of disease following exposure to a heterologous strain of equine influenza virus.

The heterologous strain tested was A/equine/2/Kentucky/98 [H3N8](obtained from Tom Chambers, University of Kentucky). Eight ponies aged 5 to 7 months were used for this efficacy study. The horses were assigned to two groups, one group of 4 to be vaccinated and another group of 4 to serve as non-vaccinated controls. Ponies were vaccinated as described in Example 8, on Day 0.

Clinical observations were performed for the vaccinates on Study Day 0 (before vaccination and at 4 hours post-vaccination), as well as on Days 1 to 8, 23, 30 to 50, and 57 post-vaccination. Controls were observed clinically on Days 29 to 50 and 57. The observations were performed and scored as described in Example 8.

The challenge material *i.e.* equine flu strain from Kentucky/98, was prepared by passing the isolated virus two times in eggs. The inoculum for each horse was prepared by thawing 0.5 ml of the virus, then diluting in 4.5 ml of sterile phosphate-buffered saline. The

inoculum was administered by nebulization using a mask for each individual horse on Day 36 post-vaccination.

The clinical observation scores were summed on each day for each horse, and horses were ranked according to the cumulative total score from days 1 to 9 post-challenge. Theses results are shown in Table 24.

TABLE 24: Clinical sign observations: total scores, ranked by total score.

Group	Halter Identity	Total Score[#] Days 1 to 9 post-challenge
1-Vaccinate	50	0
1-Vaccinate	52	0
1-Vaccinate	55	1
1-Vaccinate	15	2
2-Control	61	21
2-Control	20	25
2-Control	7	26
2-Control	13	26

[#]Total scores represent the sum of daily scores (where daily scores equal the sum of scores for coughing, nasal discharge, respiration, and depression) and are ranked from the lowest (least severe) to highest (most severe) scores.

The results of Table 24 show that the scores for vaccinates were between 0 and 2, which was significantly lower than the score for controls, which were between 21 and 26.

Rectal temperatures were measured daily beginning 6 days prior to challenge, and continuing until 9 days post-challenge. Day 0 is the day relative to challenge. Data from days 0 through 9 were included in the analysis. These results are shown in Table 25.

TABLE 25: Effect of Challenge on daily mean temperatures (°C) in vaccinated and control horses.

Day post challenge	control	vaccinate	difference
0	99.7	99.5	0.2
1	100.0	99.6	0.4
2	103.9	100.2	3.7
3	99.8	99.2	0.6
4	99.6	99.1	0.5
5	99.8	99.3	0.5
6	99.6	99.3	0.3
7	99.3	99.0	0.3
8	99.7	99.6	0.1
9	99.5	99.1	0.4

The temperatures of the control horses were higher than the temperatures of the vaccinated horses on all days. The temperature in control horses was significantly higher on day 2.

Nasopharyngeal swabs were collected on days 1 and 8, post-challenge, as described in Example 3. These samples were tested for shed virus by an egg infectivity assay as described in Example 1. The results of the assay are shown in Table 26.

TABLE 26: Virus shedding post-challenge detected by egg infectivity.

Study day		**35**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	
Days post-challenge		**-1**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	
Group	**Identity No.**	**Detection of virus***									**No. days positive per horse**
Vaccinates	**15**	0	2	0	3	3	0	2	1	0	5
	50	0	0	0	0	0	1	0	0	0	1
	52	0	0	3	3	2	2	0	0	0	4
	55	0	2	3	1	3	0	0	0	0	4
No. horses positive per day		0	2	2	3	3	2	1	1	0	
Controls	**07**	0	3	3	3	3	3	3	1	0	7
	13	0	3	3	3	3	3	3	1	0	7
	20	0	2	3	3	3	3	3	1	0	7
	61	0	3	3	3	3	3	3	2	0	7
No. horses positive per day		0	4	4	4	4	4	4	4	0	

*Values refer to the number of eggs testing positive of 3 eggs tested per sample. For statistical analysis, a sample was considered positive for virus if at least 1 egg was positive per sample.

The results of Table 26 show that the number of horses positive per day was higher for the controls than for the vaccinates. Additionally, control horses were positive for more days than vaccinates.

The scores from clinical signs relevant to influenza and the objective temperature measurements both demonstrated significant differences in the group of vaccinates when compared to the control group; this shows that the vaccine conferred significant protection from disease caused by the heterologous strain Kentucky/98.

The ability of horses to shed influenza virus post-challenge was also significantly reduced in vaccinates as opposed to controls in the mean number of days of shedding per horse. This decreased shedding by vaccinates is important in that it should serve to reduce the potential for exposure of susceptible animals to the wild-type virus in an outbreak of influenza.

Overall, the results of this study show that the vaccine safely conferred protection to a heterologous challenge by a recent and clinically relevant isolate. When the results of this study are viewed in the light of the protection previously demonstrated against heterologous challenge with a Eurasian strain (Example 9), there is clear evidence to support the assertion that this modified live vaccine can confer protection against heterologous as well as homologous equine influenza infection.

Example 11

This example describes the cloning and sequencing of equine influenza M (matrix) protein nucleic acid molecules for wild type and cold-adapted equine influenza viruses.

A. Nucleic acid molecules encoding wild type or cold-adapted equine influenza virus M protein, were produced as follows. A PCR product containing an equine M gene was produced by PCR amplification from equine influenza virus DNA, and primers w584 and w585, designated SEQ ID NO:26, and SEQ ID NO:27, respectively. A nucleic acid molecule of 1023 nucleotides, denoted $nei_{wt}M_{1023}$, with a coding strand having a nucleic acid sequence designated SEQ ID NO:1 was produced by further PCR amplification using the

above described PCR product as a template and cloned into pCR 2.1®TA cloning vector, available from Invitrogen, Carlsbad, CA, using standard procedures recommended by the manufacturer. The primers used were the T7 primer, designated by SEQ ID NO:29 and the REV primer, designated by SEQ ID NO:28. Plasmid DNA was purified using a mini-prep method available from Qiagen, Valencia, CA. PCR products were prepared for sequencing using a PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit, a PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction kit, or a PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction kit, all available from PE Applied Biosystems, Foster City, CA, following the manufacturer's protocol. Specific PCR conditions used with the kit were a rapid ramp to 95°C, hold for 10 seconds followed by a rapid ramp to 50°C with a 5 second hold then a rapid ramp to 60°C with a 4 minute hold, repeating for 25 cycles. Different sets of primers were used in different reactions: T7 and REV were used in one reaction; w584 and w585 were used in a second reaction; and efM-a1, designated SEQ ID NO:31 and efM-s1, designated SEQ ID NO:30 were used in a third reaction. PCR products were purified by ethanol/magnesium chloride precipitation. Automated sequencing of DNA samples was performed using an ABI PRISM™ Model 377 with XL upgrade DNA Sequencer, available from PE Applied Biosystems.

Translation of SEQ ID NO:1 indicates that nucleic acid molecule $nei_{wt}M_{1023}$ encodes a full-length equine influenza M protein of about 252 amino acids, referred to herein as $Pei_{wt}M_{252}$, having amino acid sequence SEQ ID NO:2, assuming an open reading frame in which the initiation codon spans from nucleotide 25 through nucleotide 28 of SEQ ID NO:1 and the termination codon spans from nucleotide 781 through nucleotide 783 of SEQ ID NO:1. The region encoding $Pei_{wt}M_{252}$, designated $nei_{wt}M_{756}$, and having a coding strand comprising nucleotides 25 to 780 of SEQ ID NO:1, is represented by SEQ ID NO:3.

SEQ ID NO:1 and SEQ ID NO:3 represent the consensus sequence obtained from two wild type nucleic acid molecules, which differ in one nucleotide. Nucleotide 663 of $nei_{wt1}M_{1023}$, *i.e.*, nucleotide 649 of $nei_{wt1}M_{756}$, was adenine, while nucleotide 663 of

$nei_{wt2}M_{1023}$, *i.e.*, nucleotide 649 of $nei_{wt2}M_{756}$, was guanine. Translation of these sequences does not result in an amino acid change at the corresponding amino acid; both translate to valine at residue 221 in $Pei_{wt}M_{252}$.

B. A nucleic acid molecule of 1023 nucleotides encoding a cold-adapted equine influenza virus M, denoted $nei_{ca1}M_{1023}$, with a coding strand having a sequence designated SEQ ID NO:4 was produced by further PCR amplification and cloned into the pCR®-Blunt cloning vector available from Invitrogen, using conditions recommended by the manufacturer, and primers T7 and REV. Plasmid DNA purification and cycle sequencing were performed as described in Example 11, part A. Translation of SEQ ID NO:4 indicates that nucleic acid molecule $nei_{ca1}M_{1023}$ encodes a full-length equine influenza M protein of about 252 amino acids, referred to herein as $Pei_{ca1}M_{252}$, having amino acid sequence SEQ ID NO:5, assuming an open reading frame in which the initiation codon spans from nucleotide 25 through nucleotide 28 of SEQ ID NO:4 and the termination codon spans from nucleotide 781 through nucleotide 783 of SEQ ID NO:4. The region encoding $Pei_{ca1}M_{252}$, designated $nei_{ca1}M_{756}$, and having a coding strand comprising nucleotides 25 to 780 of SEQ ID NO:4, is represented by SEQ ID NO:6. PCR amplification of a second nucleic acid molecule encoding a cold-adapted equine influenza M protein in the same manner resulted in molecules $nei_{ca2}M_{1023}$, identical to $nei_{ca1}M_{1023}$, and $nei_{ca2}M_{756}$, identical to $nei_{ca1}M_{756}$.

C. Comparison of the nucleic acid sequences of the coding strands of $nei_{wt}M_{1023}$ (SEQ ID NO:1) and $nei_{ca1}M_{1023}$ (SEQ ID NO:4) by DNA alignment reveals the following differences: a G to T shift at base 67, a C to T shift at base 527, and a G to C shift at base 886. Comparison of the amino acid sequences of proteins $Pei_{wt}M_{252}$ (SEQ ID NO:2) and $Pei_{ca1}M_{252}$ (SEQ ID NO:5) reveals the following differences: a V to L shift at amino acid 23 relating to the G to T shift at base 67 in the DNA sequences; and a T to I shift at amino acid 187 relating to the C to T shift at base 527 in the DNA sequences.

Example 12

This example describes the cloning and sequencing of equine influenza HA (hemagglutinin) protein nucleic acid molecules for wild type or cold-adapted equine influenza viruses.

A. Nucleic acid molecules encoding wild type or cold-adapted equine influenza virus HA proteins were produced as follows. A PCR product containing an equine HA gene was produced by PCR amplification from equine influenza virus DNA and primers w578 and w579, designated SEQ ID NO:32 and SEQ ID NO:33, respectively. A nucleic acid molecule of 1762 nucleotides encoding a wild-type HA protein, denoted $nei_{wt}HA_{1762}$, with a coding strand having a nucleic acid sequence designated SEQ ID NO:7 was produced by further PCR amplification using the above-described PCR product as a template and cloned into pCR 2.1®TA cloning vector as described in Example 11A. Plasmid DNA was purified and sequenced as in Example 11A, except that primers used in the sequencing kits were either T7 and REV in one case, or HA-1, designated SEQ ID NO:34, and HA-2, designated SEQ ID NO:35, in a second case.

Translation of SEQ ID NO:7 indicates that nucleic acid molecule $nei_{wt}HA_{1762}$ encodes a full-length equine influenza HA protein of about 565 amino acids, referred to herein as $Pei_{wt}HA_{565}$, having amino acid sequence SEQ ID NO:8, assuming an open reading frame in which the initiation codon spans from nucleotide 30 through nucleotide 33 of SEQ ID NO:7 and the termination codon spans from nucleotide 1725 through nucleotide 1727 of SEQ ID NO:7. The region encoding $Pei_{wt}HA_{565}$, designated $nei_{wt}HA_{1695}$, and having a coding strand comprising nucleotides 30 to 1724 of SEQ ID NO:7 is represented by SEQ ID NO:9.

B. A nucleic acid molecule of 1762 nucleotides encoding a cold-adapted equine influenza virus HA protein, denoted $nei_{ca1}HA_{1762}$, with a coding strand having a sequence designated SEQ ID NO:10 was produced as described in Example 11B. Plasmid DNA purification and cycle sequencing were performed as described in Example 12, part A.

Translation of SEQ ID NO:10 indicates that nucleic acid molecule $nei_{ca1}HA_{1762}$ encodes a full-length equine influenza HA protein of about 565 amino acids, referred to herein as $Pei_{ca1}HA_{565}$, having amino acid sequence SEQ ID NO:11, assuming an open reading frame in which the initiation codon spans from nucleotide 30 through nucleotide 33 of SEQ ID NO:10 and the termination codon spans from nucleotide 1725 through nucleotide 1727 of SEQ ID NO:10. The region encoding $Pei_{ca1}HA_{565}$, designated $nei_{ca1}HA_{1695}$, and having a coding strand comprising nucleotides 30 to 1724 of SEQ ID NO:10, is represented by SEQ ID NO:12.

PCR amplification of a second nucleic acid molecule encoding a cold-adapted equine influenza HA protein in the same manner resulted in molecules $nei_{ca2}HA_{1762}$, identical to $nei_{ca1}HA_{1762}$, and $nei_{ca2}HA_{1695}$, identical to $nei_{ca1}HA_{1695}$.

C. Comparison of the nucleic acid sequences of the coding strands of $nei_{wt}HA_{1762}$ (SEQ ID NO:7) and $nei_{ca1}HA_{1762}$ (SEQ ID NO:10) by DNA alignment reveals the following differences: a C to T shift at base 55, a G to A shift at base 499, a G to A shift at base 671, a C to T shift at base 738, a T to C shift at base 805, a G to A shift at base 1289, and an A to G shift at base 1368. Comparison of the amino acid sequences of proteins $Pei_{wt}HA_{565}$ (SEQ ID NO:8) and $Pei_{ca1}HA_{565}$ (SEQ ID NO:11) reveals the following differences: a P to L shift at amino acid 18 relating to the C to T shift at base 55 in the DNA sequences; a G to E shift at amino acid 166 relating to the G to A shift at base 499 in the DNA sequences; an R to W shift at amino acid 246 relating to the C to T shift at base 738 in the DNA sequences; an M to T shift at amino acid 268 relating to the T to C shift at base 805 in the DNA sequences; a K to E shift at amino acid 456 relating to the A to G shift at base 1368 in the DNA sequences. There is no change of the serine (S) at residue 223 relating to the G to A shift at base 671 in the DNA sequences, nor is there a change of the arginine (R) at residue 429 relating to the G to A shift at base 1289 in the DNA sequences.

Example 13

This example describes the cloning and sequencing of equine influenza PB2 protein (RNA-directed RNA polymerase) nucleic acid molecules corresponding to the N-terminal portion of the protein, for wild type or cold-adapted equine influenza viruses.

A. Nucleic acid molecules encoding wild type or cold-adapted equine influenza virus PB2-N proteins were produced as follows. A PCR product containing an N-terminal portion of the equine PB2 gene was produced by PCR amplification from equine influenza virus DNA, and primers w570 and w571, designated SEQ ID NO:36 and SEQ ID NO:37, respectively. A nucleic acid molecule of 1241 nucleotides encoding a wild type PB2-N protein, denoted $nei_{wt}PB2\text{-}N_{1241}$, with a coding strand having a nucleic acid sequence designated SEQ ID NO:13 was produced by further PCR amplification using the above described PCR product as a template and cloned as described in Example 11B. Plasmid DNA was purified and sequenced as in Example 11B, except that only T7 and REV primers were used in the sequencing kits.

Translation of SEQ ID NO:13 indicates that nucleic acid molecule $nei_{wt}PB2\text{-}N_{1241}$ encodes an N-terminal portion of influenza PB2 protein of about 404 amino acids, referred to herein as $P_{wt}PB2\text{-}N_{404}$, having amino acid sequence SEQ ID NO:14, assuming an open reading frame in which the initiation codon spans from nucleotide 28 through nucleotide 30 of SEQ ID NO:13, and the last codon spans from nucleotide 1237 through nucleotide 1239. The region encoding $P_{wt}PB2\text{-}N_{404}$, designated $nei_{wt}PB2\text{-}N_{1214}$, and having a coding strand comprising nucleotides 28 to 1239of SEQ ID NO:13 is represented by SEQ ID NO:15.

B. A nucleic acid molecule of 1239 nucleotides encoding an N-terminal portion of influenza PB2 cold-adapted equine influenza virus PB2-N protein, denoted $nei_{cal}PB2\text{-}N_{1241}$, with a coding strand having a sequence designated SEQ ID NO:16 was produced, and sequenced as described in as in Example 12, part A.

Translation of SEQ ID NO:16 indicates that nucleic acid molecule $nei_{cal}PB2\text{-}N_{1241}$ encodes an N-terminal portion of equine influenza PB-2 protein of about 404 amino acids,

referred to herein as P_{ca1}PB2-N_{404}, having amino acid sequence SEQ ID NO:17, assuming an open reading frame in which the initiation codon spans from nucleotide 28 through nucleotide 30 of SEQ ID NO:16, and the last codon spans from nucleotide 1237 through nucleotide 1239. The region encoding P_{ca1}PB2-N_{404}, designated nei_{ca1}PB2-N_{1214}, and having a coding strand comprising nucleotides 28 to 1239 of SEQ ID NO:16, is represented by SEQ ID NO:18.

PCR amplification of a second nucleic acid molecule encoding a cold-adapted equine influenza PB2-N protein in the same manner resulted in molecules nei_{ca2}PB2-N_{1241}, identical to nei_{ca1}PB2-N_{1241}, and nei_{ca2}PB2-N_{1214}, identical to nei_{ca1}PB2-N_{1214}.

C. Comparison of the nucleic acid sequences of the coding strands of nei_{wt}PB2-N_{1241} (SEQ ID NO:13) and nei_{ca1}PB2-N_{1241} (SEQ ID NO:16) by DNA alignment reveals the following difference: a T to C base shift at base 370. Comparison of the amino acid sequences of proteins P_{wt}PB2-N_{404} (SEQ ID NO:14) and P_{ca1}PB2-N_{404} (SEQ ID NO:17) reveals the following difference: a Y to H shift at amino acid 124 relating to the a T to C shift at base 370 in the DNA sequence.

Example 14

This example describes the cloning and sequencing of equine influenza PB2 protein (RNA-directed RNA polymerase) nucleic acid molecules corresponding to the C-terminal portion of the protein, for wild type or cold-adapted equine influenza viruses.

A. Nucleic acid molecules encoding wild type or cold-adapted equine influenza virus PB2-C proteins were produced as follows. A PCR product containing the C-terminal portion of the equine PB2 gene was produced by PCR amplification using from equine influenza virus DNA and primers w572 and w573, designated SEQ ID NO:38 and SEQ ID NO:39, respectively. A nucleic acid molecule of 1233 nucleotides encoding a wild type PB2-C protein, denoted nei_{wt}PB2-C_{1233}, with a coding strand having a nucleic acid sequence designated SEQ ID NO:19 was produced by further PCR amplification using the above-described PCR product as a template and cloned as described in Example 11B. Plasmid

DNA was purified and sequenced as in Example 11A, except that different primers were used in the sequencing kits. T7 and REV were used in one instance; efPB2-a1, designated SEQ ID NO:40 and efPB2-s1, designated SEQ ID NO:41 were used in another instance, and efPB2-a2, designated SEQ ID NO:42 and efPB2-s2, designated SEQ ID NO:43 were used in another instance.

Translation of SEQ ID NO:19 indicates that nucleic acid molecule $nei_{wt1}PB2\text{-}C_{1233}$ encodes a C-terminal portion of influenza PB2 protein of about 398 amino acids, referred to herein as $P_{wt}PB2\text{-}C_{398}$, having amino acid sequence SEQ ID NO:20, assuming an open reading frame having a first codon spans from nucleotide 3 through nucleotide 5 and a termination codon which spans from nucleotide 1197 through nucleotide 1199 of SEQ ID NO:19. Because SEQ ID NO:19 is only a partial gene sequence, it does not contain an initiation codon. The region encoding $P_{wt}PB2\text{-}C_{398}$, designated $nei_{wt}PB2\text{-}C_{1194}$, and having a coding strand comprising nucleotides 3 to 1196 of SEQ ID NO:19 is represented by SEQ ID NO:21.

PCR amplification of a second nucleic acid molecule encoding a wild type equine influenza PB2-N protein in the same manner resulted in a nucleic acid molecule of 1232 nucleotides denoted $nei_{wt2}PB2\text{-}N_{1232}$, with a coding strand with a sequence designated SEQ ID NO:22. $nei_{wt2}PB2\text{-}N_{1232}$ is identical to $nei_{wt1}PB2\text{-}C_{1233}$, expect that $nei_{wt2}PB2\text{-}N_{1232}$ lacks one nucleotide on the 5'-end. Translation of SEQ ID NO:22 indicates that nucleic acid molecule $nei_{wt1}PB2\text{-}C_{1233}$ also encodes $P_{wt}PB2\text{-}C_{398}$ (SEQ ID NO:20), assuming an open reading frame having a first codon which spans from nucleotide 2 through nucleotide 4 and a termination codon spans from nucleotide 1196 through nucleotide 1198 of SEQ ID NO:22. Because SEQ ID NO:22 is only a partial gene sequence, it does not contain an initiation codon. The nucleic acid molecule having a coding strand comprising nucleotides 2 to 1195 of SEQ ID NO:22, denoted $nei_{wt2}PB2\text{-}C_{1194}$, is identical to SEQ ID NO:21.

B. A nucleic acid molecule of 1232 nucleotides encoding a C-terminal portion of influenza PB2 cold-adapted equine influenza virus protein, denoted $nei_{ca1}PB2\text{-}C_{1232}$, and

having a coding strand having a sequence designated SEQ ID NO:23 was produced as described in as in Example 14, part A, except that the pCR®-Blunt cloning vector was used.

Translation of SEQ ID NO:23 indicates that nucleic acid molecule $nei_{ca1}PB2\text{-}C_{1232}$ encodes a C-terminal portion of equine influenza PB-2 protein of about 398 amino acids, referred to herein as $P_{ca1}PB2\text{-}C_{398}$, having amino acid sequence SEQ ID NO:24, assuming an open reading frame having a first codon which spans from nucleotide 2 through nucleotide 4 and a termination codon spans from nucleotide 1196 through nucleotide 1198 of SEQ ID NO:23. Because SEQ ID NO:23 is only a partial gene sequence, it does not contain an initiation codon. The region encoding $P_{ca1}PB2\text{-}C_{398}$, designated $nei_{ca1}PB2\text{-}C1194$, and having a coding strand comprising nucleotides 2 to 1195 of SEQ ID NO:23, is represented by SEQ ID NO:25.

PCR amplification of a second nucleic acid molecule encoding a cold-adapted equine influenza PB2-C protein in the same manner resulted in molecules $nei_{ca2}PB2\text{-}C_{1231}$, containing one less nucleotide at the 3'end than $nei_{ca1}PB2\text{-}N_{1241}$; and $nei_{ca2}PB2\text{-}N_{1214}$, identical to $nei_{ca1}PB2\text{-}N_{1214}$.

C. Comparison of the nucleic acid sequences of the coding strands of $nei_{wt1}PB2\text{-}C_{1233}$ (SEQ ID NO:19) and $nei_{ca1}PB2\text{-}C_{1232}$ (SEQ ID NO:23) by DNA alignment reveals the following differences: an A to C base shift at base 153 of SEQ ID NO:19, and a G to A base shift at base 929 of SEQ ID NO:19. Comparison of the amino acid sequences of proteins $P_{wt}PB2\text{-}C_{398}$ (SEQ ID NO:20) and $P_{ca1}PB2\text{-}_{398}$ (SEQ ID NO:24) reveals the following difference: a K to Q shift at amino acid 51 when relating to the an A to C base shift at base 153 in the DNA sequences. There is no amino acid shift resulting from the G to A base shift at base 929.

Example 15

This example describes the cloning and sequencing of equine influenza PB2 protein (RNA-directed RNA polymerase) nucleic acid molecules for wild type or cold-adapted equine influenza viruses.

A. Nucleic acid molecules encoding wild type or cold-adapted equine influenza virus PB2 proteins were produced as follows. The wild type or cold-adapted equine influenza genes were cloned in two fragments, the N-terminal portion was produced as in Example 13 and the C-terminal portion of the gene was produced as in Example 14.

The DNA sequence for the wild type equine influenza PB2 gene was generated by combining the consensus sequences for the wild type PB2-N protein , denoted nei_{wt}PB2-N_{1241} (SEQ ID NO:13) with the gene fragments for the wild type PB2-C protein, denoted nei_{wt1}PB2-C_{1233} (SEQ ID NO:19) and nei_{wt2}PB2-C_{1232} (SEQ ID NO: 22). The result of combining the consensus sequences from the N-terminal and C-terminal portions of the PB2 wild type influenza virus yielded a complete DNA sequence denoted $nei_{wt}PB2_{2341}$(SEQ ID NO:44). Translation of SEQ ID NO:44 indicates that the nucleic acid molecule $nei_{wt}PB2_{2341}$ encodes a full length equine influenza PB2 protein of about 759 amino acids referred to herein as $Pei_{wt}PB2_{759}$, having amino acid sequence SEQ ID NO: 45, assuming an open reading frame in which the initiation codon spans from nucleotide 28 through nucleotide 30 of SEQ ID NO: 44 and the termination codon spans from nucleotide 2305 through nucleotide 2307 of SEQ ID NO: 44. The region encoding $Pei_{wt}PB2_{759}$, designated $nei_{wt}PB2_{2277}$, and having a coding strand comprising nucleotides 28 to 2304 of SEQ ID NO: 44, is SEQ ID NO: 46.

B. A DNA sequence of 2341 nucleotides encoding a cold-adapted equine influenza virus PB2 , denoted $nei_{ca1}PB2_{2341}$, with a sequence denoted SEQ ID NO: 47 was produced by combining the sequences for the N-terminal and C-terminal portions of the PB2 cold-adapted equine influenza gene. The clones for the N-terminal sequences are denoted nei_{ca1}PB2-N_{1241} and nei_{ca2}PB2-N_{1241} which are identical and are represented by SEQ ID NO:16. The clones for the C-terminal sequences are denoted nei_{ca1}PB2-C_{1232} and nei_{ca2}PB2-C_{1231}, represented by SEQ ID NO:23.

Translation of SEQ ID NO:47 indicates that nucleic acid molecule $nei_{ca1}PB2_{2341}$ encodes a full-length equine influenza PB2 protein of about 759 amino acids, referred to

herein as $Pei_{cal}PB2_{759}$, having amino acid sequence SEQ ID NO:48, assuming an open reading frame in which the initiation codon spans from nucleotide 28 through nucleotide 30 of SEQ ID NO: 47 and the termination codon spans from nucleotide 2305 through nucleotide 2307 of SEQ ID NO:47. The region encoding $Pei_{cal}PB2_{759}$ designated $nei_{cal}PB2_{2277}$ and having a coding strand comprising nucleotides 28 to 2304 of SEQ ID NO:49.

C. Comparison of the nucleic acid sequences of the coding strands of $nei_{wt}PB_{2341}$ (SEQ ID NO:44) and $nei_{cal}PB2_{2341}$ (SEQ ID NO:47) by DNA alignment reveals the following differences: a T to C base shift at base 370, an A to C base shift at base 1261, and a G to A base shift at base 2037. Comparison of the amino acid sequences of proteins $Pei_{wt}PB2_{759}$ (SEQ ID NO:45) and $Pei_{cal}PB2_{759}$ (SEQ ID NO:48) reveals the following differences: a Y to H shift at amino acid 124 relating to the a T to C shift at base 370 in the DNA sequence, a K to Q shift at amino acid 421 relating to the A to C shift at base 1261 in the DNA sequence. The third nucleotide shift at base 2037 does not result in an amino acid shift at amino.

Example 16

This example describes the cloning and sequencing of equine influenza NS (nonstructural) protein nucleic acid molecules for wild type or cold-adapted equine influenza viruses.

A. Nucleic acid molecules encoding wild type or cold-adapted equine influenza virus NS proteins were produced as follows. A PCR product containing an equine NS gene was produced by PCR amplification from equine influenza virus DNA and primers w586 and w587, designated SEQ ID NO:59 and SEQ ID NO:60, respectively. A nucleic acid molecule of 891 nucleotides encoding a wild-type NS protein, denoted $nei_{wt}NS_{891}$, with a coding strand having a nucleic acid sequence designated SEQ ID NO:50 was produced by further PCR amplification using the above-described PCR product as a template and cloned into pCR 2.1®TA cloning vector as described in Example 11A. Plasmid DNA was purified and

sequenced as in Example 11A, except that primers used in the sequencing kits were only T7 and REV were used in the sequencing kits.

Translation of SEQ ID NO:50 indicates that nucleic acid molecule $nei_{wt1}NS_{891}$ encodes a full-length equine influenza NS protein of about 230 amino acids, referred to herein as $Pei_{wt1}NS_{230}$, having amino acid sequence SEQ ID NO:51, assuming an open reading frame in which the initiation codon spans from nucleotide 27 through nucleotide 29 of SEQ ID NO:50 and the termination codon spans from nucleotide 717 through nucleotide 719 of SEQ ID NO:50. The region encoding $Pei_{wt1}NS_{230}$, designated $nei_{wt1}NS_{690}$, and having a coding strand comprising nucleotides 27 to 716 of SEQ ID NO:50 is represented by SEQ ID NO:52.

PCR amplification of a second nucleic acid molecule encoding a wild type equine influenza NS protein in the same manner resulted in molecules $nei_{wt2}NS_{891}$, identical to $nei_{wt1}NS_{891}$ in the coding region; i.e. $nei_{wt2}NS_{690}$,is identical to $nei_{wt1}NS_{690}$. $nei_{wt2}NS_{891}$ differs from $nei_{wt1}NS_{891}$ in one nucleotide at base 827 (G to A) which is 111 bases downstream from the stop codon. PCR amplification of a third nucleic acid encoding a wild type equine influenza NS protein in the same manner resulted in a nucleic acid molecule of 888 nucleotides denoted $nei_{wt3}NS_{888}$, with a coding strand with a nucleic acid sequence designated SEQ ID NO: 53. $nei_{wt3}NS_{888}$ is identical to $nei_{wt1}NS_{891}$, except that $nei_{wt3}NS_{888}$, lacks two nucleotides on the 5' end and one nucleotide on the 3' end. Translation of SEQ ID NO:53 indicates that nucleic acid molecule $nei_{wt3}NS_{888}$ also encodes $Pei_{wt1}NS_{230}$ (SEQ ID NO:51), assuming an open reading frame having an initiation codon which spans from nucleotide 25 through nucleotide 27 of SEQ ID NO: 53 and a termination codon which spans from nucleotide 715 through nucleotide 717 of SEQ ID NO:53. The nucleic acid molecule having a coding strand comprising nucleotides 25 to 714 of SEQ ID 53, denoted $nei_{wt3}NS_{690}$, is identical to SEQ ID NO:52.

PCR amplification of a fourth nucleic acid of 468 nucleotides encoding a C-terminal portion of the wild type equine influenza NS protein, denoted $nei_{wt4}NS_{468}$ and having a

coding sequence designated SEQ ID NO:54 was produced. Translation of SEQ ID NO:54 indicates that nucleic acid molecule $nei_{wt4}NS_{468}$ encodes a C-terminal portion of equine influenza NS protein of about 97 amino acids, referred to herein as $Pei_{wt4}NS_{97}$, having amino acid sequence SEQ ID NO:55, assuming an open reading frame having a first codon which spans from nucleotide 3 to 5 of SEQ ID NO: 54, and a termination codon spans from nucleotide 294 through 296 of SEQ ID NO:54. Because SEQ ID NO:54 is only a partial gene sequence, it does not contain an initiation codon. The region encoding $Pei_{wt4}NS_{97}$, designated $nei_{wt4}NS_{293}$, and having a coding strand comprising nucleotides 1 to 293 of SEQ ID NO:54, is represented by SEQ ID NO: 56.

B. A nucleic acid molecule of 888 nucleotides encoding a cold-adapted equine influenza virus NS protein, denoted $nei_{ca1}NS_{888}$, with a coding strand having a sequence designated SEQ ID NO:57 was produced and sequenced as described in Example 16, part A.

Translation of SEQ ID NO:57 indicates that nucleic acid molecule $nei_{ca1}NS_{888}$ encodes a full-length equine influenza NS protein of about 230 amino acids, referred to herein as $Pei_{ca1}NS_{230}$, having amino acid sequence SEQ ID NO:58, assuming an open reading frame in which the initiation codon spans from nucleotide 27 through nucleotide 29 of SEQ ID NO:57 and the termination codon spans from nucleotide 717 through nucleotide 719 of SEQ ID NO:57. The region encoding $Pei_{ca1}NS_{230}$, designated $nei_{ca1}NS_{690}$, and having a coding strand comprising nucleotides 27 to 716 of SEQ ID NO:57, is represented by SEQ ID NO:59.

PCR amplification of a second nucleic acid molecule encoding a cold-adapted equine influenza NS protein in the same manner resulted in molecules $nei_{ca2}NS_{887}$, containing one less nucleotide at the 3' end than $nei_{ca1}NS_{888}$; the coding region $nei_{ca2}NS_{690}$ is identical to $nei_{ca1}NS_{690}$.

C. Comparison of the nucleic acid sequences of the coding strands of $nei_{wt}NS_{891}$ (SEQ ID NO:50) and $nei_{ca1}NS_{888}$ (SEQ ID NO:57) by DNA alignment reveals the following difference: an A to G shift at base 827 which is 111 bases downstream from the stop codon.

The 3' fragment encoding $nei_{wt4}NS_{468}$ (SEQ ID NO:54) has one shift T to C found at base 633 relative to the full-length consensus sequence. Comparison of the amino acid sequences of proteins $Pei_{wt}NS_{230}$ (SEQ ID NO:51) and $Pei_{ca1}NS_{230}$ (SEQ ID NO:58) reveals that there are no differences between amino acid sequences of the wild type and cold-adapted proteins.

Example 17

This example describes the cloning and sequencing of equine influenza PB1 protein (RNA-directed RNA polymerase 1) nucleic acid molecules corresponding to the N-terminal portion of the protein, for wild type or cold-adapted equine influenza viruses.

A. Nucleic acid molecules encoding wild type or cold-adapted equine influenza virus PB1-N proteins were produced as follows. A PCR product containing an N-terminal portion of the equine PB1 gene was produced by PCR amplification from equine influenza virus DNA, and primers T7 and REV. A nucleic acid molecule of 1229 nucleotides encoding a wild type PB1-N protein, denoted nei_{wt1}PB1-N_{1229}, with a coding strand having a nucleic acid sequence designated SEQ ID NO:62 was produced by further PCR amplification using the above described PCR product as a template and cloned as described in Example 11B. Plasmid DNA was purified and sequenced as in Example 11B, except that only T7 and REV primers were used in the sequencing kits.

Translation of SEQ ID NO:62 indicates that nucleic acid molecule nei_{wt1}PB1-N_{1229} encodes an N-terminal portion of influenza PB1 protein of about 398 amino acids, referred to herein as Pei_{wt1}PB1-N_{398}, having amino acid sequence SEQ ID NO:63, assuming an open reading frame in which the initiation codon spans from nucleotide 36 through nucleotide 38 of SEQ ID NO:62, and the last codon spans from nucleotide 1227 through nucleotide 1229 of SEQ ID NO:62. The region encoding Pei_{wt1}PB1-N_{398}, designated nei_{wt1}PB1-N_{1194}, and having a coding strand comprising nucleotides 36 to 1229 of SEQ ID NO:62 is represented by SEQ ID NO:64.

PCR amplification of a second nucleic acid molecule encoding a wild type equine influenza PB1-N protein in the same manner resulted in a nucleic acid molecule of 673

nucleotides denoted $nei_{wt2}PB1\text{-}N_{673}$, with a coding strand with a sequence designated SEQ ID NO:65. Translation of SEQ ID NO:65 indicates that nucleic acid molecule $nei_{wt2}PB1\text{-}N_{673}$ encodes $Pei_{wt2}PB1\text{-}N_{212}$ (SEQ ID NO:66), assuming an open reading frame having an initiation codon which spans from nucleotide 36 through nucleotide 38 of SEQ ID NO:65 and a last codon which spans from nucleotide 671 through nucleotide 673 of SEQ ID NO:65. Because SEQ ID NO:65 is only a partial gene sequence, it does not contain a stop codon. The nucleic acid molecule having a coding strand comprising nucleotides 36 to 671 of SEQ ID NO:65, denoted $nei_{wt2}PB1\text{-}N_{636}$, is designated SEQ ID NO:67.

B. A nucleic acid molecule of 1225 nucleotides encoding an N-terminal portion of influenza PB1 cold-adapted equine influenza virus PB1-N protein, denoted $nei_{ca1}PB1\text{-}N_{1225}$, with a coding strand having a sequence designated SEQ ID NO:68 was produced, and sequenced as described in as in Example 17, part A.

Translation of SEQ ID NO:68 indicates that nucleic acid molecule $nei_{ca1}PB1\text{-}N_{1225}$ encodes an N-terminal portion of equine influenza PB-1 protein of about 395 amino acids, referred to herein as $Pei_{ca1}PB1\text{-}N_{395}$, having amino acid sequence SEQ ID NO:69, assuming an open reading frame in which the initiation codon spans from nucleotide 34 through nucleotide 36 of SEQ ID NO:68, and a last codon which spans from nucleotide 1216 through nucleotide 1218 of SEQ ID NO:68. The region encoding $Pei_{ca1}PB1\text{-}N_{395}$, designated $nei_{ca1}PB1\text{-}N_{1185}$, and having a coding strand comprising nucleotides 34 to 1218 of SEQ ID NO:68, is represented by SEQ ID NO:70.

PCR amplification of a second nucleic acid molecule encoding a cold-adapted equine influenza PB1-N protein in the same manner resulted in molecules $nei_{ca2}PB1\text{-}N_{1221}$, designated SEQ ID NO:71, containing four less nucleotides at the 5' end than $nei_{ca1}PB1\text{-}N_{1225}$; the coding region $nei_{ca2}PB1\text{-}N_{1185}$, is identical to $nei_{ca1}PB1\text{-}N_{1185}$.

C. Comparison of the nucleic acid sequences of the coding strands of $nei_{wt}PB1\text{-}N_{1229}$ (SEQ ID NO:62) and $nei_{ca1}PB1\text{-}N_{1225}$ (SEQ ID NO:68) by DNA alignment reveals no differences in the coding regions. Comparison of the amino acid sequences of proteins

$Pei_{wt}PB1\text{-}N_{395}$ (SEQ ID NO:63) and $Pei_{cal}PB1\text{-}N_{395}$ (SEQ ID NO:69) also reveals no differences.

Example 18

This example describes the cloning and sequencing of equine influenza PB1 protein (RNA-directed RNA polymerase1) nucleic acid molecules corresponding to the C-terminal portion of the protein, for wild type or cold-adapted equine influenza viruses.

A. Nucleic acid molecules encoding wild type or cold-adapted equine influenza virus PB1-C proteins were produced as follows. A PCR product containing an C-terminal portion of the equine PB1 gene was produced by PCR amplification from equine influenza virus DNA, and primer w569 designated SEQ ID NO:102. A nucleic acid molecule of 1234 nucleotides encoding a wild type PB1-C protein, denoted $nei_{wtl}PB1\text{-}C_{1234}$, with a coding strand having a nucleic acid sequence designated SEQ ID NO:85 was produced by further PCR amplification using the above described PCR product as a template and cloned as described in Example 11B. Plasmid DNA was purified and sequenced as in Example 11A, except that different primers were used in the sequencing kits. T7, REV, w569, efPB1-a1, designated SEQ ID NO:97, efPB1-a2, designated SEQ ID NO:98, efPB1-s1, designated SEQ ID NO: 99, efPB1-s2, designated SEQ ID NO: 100, and efPB1-s3, designated SEQ ID NO:101 were used in one instance, T7, REV, efPB1-a1, efPB1-a2,efPB1-s1, efPB1-s2, and efPB1-s3 were used in another instance and T7 and REV were used in another instance.

Translation of SEQ ID NO:85 indicates that nucleic acid molecule $nei_{wtl}PB1\text{-}C_{1234}$ encodes an C-terminal portion of influenza PB1 protein of about 396 amino acids, referred to herein as $Pei_{wtl}PB1\text{-}C_{396}$, having amino acid sequence SEQ ID NO:86, assuming an open reading frame in which the first codon spans from nucleotide 1 through nucleotide 3 of SEQ ID NO:85 and a termination codon which spans from nucleotide 1189 through nucleotide 1191 of SEQ ID NO:85. Because SEQ ID NO:85 is only a partial gene sequence, it does not contain an initiation codon. The region encoding $Pei_{wtl}PB1\text{-}C_{396}$, designated $nei_{wtl}PB1\text{-}C_{1188}$,

and having a coding strand comprising nucleotides 1 to 1188 of SEQ ID NO:85 is represented by SEQ ID NO:87.

PCR amplification of a second nucleic acid molecule encoding a wild type equine influenza PB1-C protein in the same manner resulted in a nucleic acid molecule of 1240 nucleotides denoted $nei_{wt2}PB1\text{-}C_{1240}$, with a coding strand with a sequence designated SEQ ID NO:88. Translation of SEQ ID NO:88 indicates that nucleic acid molecule $nei_{wt2}PB1\text{-}N_{1240}$ encodes a molecule designated $Pei_{wt2}PB1\text{-}C_{396}$ (SEQ ID NO: 89) which differs from $Pei_{wt1}PB1\text{-}C_{396}$ (SEQ ID NO:85) in one nucleotide. Nucleotide 382 of $nei_{wt1}PB1\text{-}C_{1234}$, i.e. nucleotide 382 of $nei_{wt1}PB1\text{-}C_{1188}$ was A, while nucleotide 389 of $nei_{wt2}PB1\text{-}C_{1240}$, i.e. nucleotide 382 of $nei_{wt2}PB1\text{-}C_{1188}$ was T. Translation of $nei_{wt2}PB1\text{-}C_{1240}$ results in an amino acid change of T to S.

B. A nucleic acid molecule of 1241 nucleotides encoding an C-terminal portion of influenza PB1 cold-adapted equine influenza virus PB1-C protein, denoted $nei_{ca1}PB1\text{-}C_{1241}$, with a coding strand having a sequence designated SEQ ID NO:91 was produced, and sequenced as described in as in Example 18, part A.

Translation of SEQ ID NO:91 indicates that nucleic acid molecule $nei_{ca1}PB1\text{-}C_{1241}$ encodes an C-terminal portion of equine influenza PB-1 protein of about 396 amino acids, referred to herein as $Pei_{ca1}PB1\text{-}C_{396}$, having amino acid sequence SEQ ID NO:92, assuming an open reading frame in which the first codon spans from nucleotide 8 through nucleotide 10 of SEQ ID NO:91 and a termination codon that spans from nucleotide 1196 through nucleotide 1198 of SEQ ID NO:91. Because SEQ ID NO:91 is only a partial gene sequence, it does not contain an initiation codon. The region encoding $Pei_{ca1}PB1\text{-}C_{396}$, designated $nei_{ca1}PB1\text{-}C_{1188}$, and having a coding strand comprising nucleotides 8 to 1195 of SEQ ID NO:91, is represented by SEQ ID NO:93.

PCR amplification of a second nucleic acid molecule encoding a cold-adapted equine influenza PB1-C protein in the same manner resulted in a nucleic acid molecule of 1241 nucleotides denoted $nei_{ca2}PB1\text{-}C_{1241}$, with a coding strand with a sequence designated SEQ

ID NO:94. Translation of SEQ ID NO:94 indicates that nucleic acid molecule $nei_{ca2}PB1$-C_{1241} encodes a molecule designated $Pei_{ca2}PB1$-C_{396} (SEQ ID NO: 95) which differs from $Pei_{ca1}PB1$-C_{396} (SEQ ID NO:92) in one nucleotide. Nucleotide 1044 of $nei_{ca1}PB1$-C_{1241}, i.e. nucleotide 1037 of $nei_{ca1}PB1$-N_{1188} was A, while nucleotide 1044 of $nei_{ca2}PB1$-C_{1241}, i.e. nucleotide 1037 of $nei_{ca2}PB1$-C_{1188} was G. Translation of $nei_{ca2}PB1$-C_{1241} results in an amino acid change of R to K.

C. Comparison of the nucleic acid sequences of the coding strands of $nei_{wt1}PB1$-C_{1234} (SEQ ID NO:85) and $nei_{ca1}PB1$-C_{1241} (SEQ ID NO:91) by DNA alignment reveals the following differences: a C to T shift at base 600 of SEQ ID NO:85, and a T to A shift at base 603 of SEQ ID NO:85. Comparison of the amino acid sequences of proteins $Pei_{wt1}PB1$-C_{396} (SEQ ID NO:86) and $Pei_{ca1}PB1$-N_{396} (SEQ ID NO:92) reveals the following difference: a H to Q amino acid shift 203 when relating to the T to A base shift at base 603 in the DNA sequences. There is no amino acid shift resulting from the C to T base shift at base 600.

Example 19

This example describes the cloning and sequencing of equine influenza PB1 protein (RNA-directed RNA polymerase) nucleic acid molecules for wild type or cold-adapted equine influenza viruses.

A. Nucleic acid molecules encoding wild type or cold-adapted equine influenza virus PB1 proteins were produced as follows. The wild type or cold-adapted equine influenza genes were cloned in two fragments, the N-terminal portion was produced as in Example 17 and the C-terminal portion of the gene was produced as in Example 18.

The DNA sequence for the wild type equine influenza PB1 gene was generated by combining the sequences for the wild type PB1-N protein , $nei_{wt1}PB1$-N_{1229} (SEQ ID NO:62) and $nei_{wt2}PB1$-N_{673} (SEQ ID NO: 65) with the gene fragments for the wild type PB1-C protein, denoted $nei_{wt1}PB1$-C_{1234} (SEQ ID NO:85) and $nei_{wt2}PB1$-C_{1240} (SEQ ID NO: 88). The result of combining the N-terminal and C-terminal portions of the PB1 wild type influenza virus yielded a complete DNA sequence of 2341 nucleotides denoted

$nei_{wt}PB1_{2341}$(SEQ ID NO:103). Translation of SEQ ID NO:103 indicates that the nucleic acid molecule $nei_{wt}PB2_{2341}$ encodes a full length equine influenza PB1 protein of about 757 amino acids referred to herein as $Pei_{wt}PB1_{757}$, having amino acid sequence SEQ ID NO:104, assuming an open reading frame in which the initiation codon spans from nucleotide 25 through nucleotide 27of SEQ ID NO: 103 and the termination codon spans from nucleotide 2293 through nucleotide 2295 of SEQ ID NO: 103. The region encoding $Pei_{wt}PB1_{757}$ designated $nei_{wt}PB1_{2271}$, and having a coding strand comprising nucleotides 25 to 2292 of SEQ ID NO: 103, is SEQ ID NO: 105.

B. A DNA sequence of 2341 nucleotides encoding a cold-adapted equine influenza virus PB1 , denoted $nei_{ca1}PB1_{2341}$, with a sequence denoted SEQ ID NO: 106 was produced by combining the sequences for the N-terminal and C-terminal portions of the PB1 cold-adapted equine influenza gene. The clones for the N-terminal sequences are denoted $nei_{ca1}PB1\text{-}N_{1225}$ (SEQ ID NO: 68) and $nei_{ca2}PB1\text{-}N_{1221}$ (SEQ ID NO: 71). The clones for the C-terminal sequences are denoted $nei_{ca1}PB1\text{-}C_{1241}$ (SEQ ID NO:91) and $nei_{ca2}PB1\text{-}C_{1241}$,(SEQ ID NO: 94).

Translation of SEQ ID NO:106 indicates that nucleic acid molecule $nei_{ca1}PB1_{2341}$ encodes a full-length equine influenza PB1 protein of about 757 amino acids, referred to herein as $Pei_{ca1}PB1_{757}$, having amino acid sequence SEQ ID NO:107, assuming an open reading frame in which the initiation codon spans from nucleotide 25 through nucleotide 27 SEQ ID NO: 106 and the termination codon spans from nucleotide 2296 through nucleotide 2298 of SEQ ID NO:106. The region encoding $Pei_{ca1}PB1_{757}$ designated $nei_{ca1}PB1_{2271}$ and having a coding strand comprising nucleotides 25 to 2295 of SEQ ID NO:108.

C. Comparison of the nucleic acid sequences of the coding strands of $nei_{wt}PB1_{2341}$ (SEQ ID NO:103) and $nei_{ca1}PB1_{2341}$ (SEQ ID NO:106) by DNA alignment reveals the following differences: a C to T base shift at base 1683, and a T to A base shift at base 1686. Comparison of the amino acid sequences of proteins $Pei_{wt}PB1_{757}$ (SEQ ID NO:104) and $Pei_{ca1}PB1_{757}$ (SEQ ID NO:107) reveals the following differences: no shift in base C at amino

acid 561 relating to the C to T shift at base 1683 , and a H to Q shift at amino acid 562 relating to the a T to A shift at base 1683 in the DNA sequence.

Example 20

This example describes the cloning and sequencing of equine influenza PA protein (RNA polymerase A) nucleic acid molecules corresponding to the C-terminal portion of the protein, for wild type or cold-adapted equine influenza viruses.

A. Nucleic acid molecules encoding wild type or cold-adapted equine influenza virus PA-C proteins were produced as follows. A PCR product containing the C-terminal portion of the equine PA gene was produced by PCR amplification using from equine influenza virus DNA and primers C+PA and C-PA, designated SEQ ID NO:83 and SEQ ID NO:84 respectively. A nucleic acid molecule of 1228 nucleotides encoding a wild type PA-C protein, denoted $nei_{wtl}PA\text{-}C_{1228}$, with a coding strand having a nucleic acid sequence designated SEQ ID NO:76 was produced by further PCR amplification using the above-described PCR product as a template and cloned as described in Example 11B. Plasmid DNA was purified and sequenced as in Example 11A, except that different primers were used in the sequencing kits. T7 and REV were used in one instance; PAC-1,designated SEQ ID NO:72, PAC-2, designated SEQ ID NO:73, PAC-3,designated SEQ ID NO:74, PAC-4, designated SEQ ID NO: 75, T7 and REV were used in another instance; and PAC-1, PAC-2, T7 and REV were used in another instance.

Translation of SEQ ID NO:76 indicates that nucleic acid molecule $nei_{wtl}PA\text{-}C_{1228}$ encodes a C-terminal portion of influenza PA protein of about 388 amino acids, referred to herein as $Pei_{wtl}PA\text{-}C_{388}$, having amino acid sequence SEQ ID NO:77, assuming an open reading frame having a first codon spans from nucleotide 3 through nucleotide 5 of SEQ ID NO:76 and a termination codon which spans from nucleotide 1167 through nucleotide 1169 of SEQ ID NO:76. Because SEQ ID NO:76 is only a partial gene sequence, it does not contain an initiation codon. The region encoding $Pei_{wtl}PA\text{-}C_{388}$, designated $nei_{wtl}PA\text{-}C_{1164}$,

and having a coding strand comprising nucleotides 3 to 1166 of SEQ ID NO:76 is represented by SEQ ID NO:78.

PCR amplification of a second nucleic acid molecule encoding a wild type equine influenza PA-C protein in the same manner resulted in a nucleic acid molecule of 1223 nucleotides denoted $nei_{wt2}PA\text{-}C_{1223}$, with a coding strand with a sequence designated SEQ ID NO:79. $nei_{wt2}PA\text{-}C_{1223}$ is identical to $nei_{wt1}PA\text{-}C_{1228}$, with the exception of a T to C base shift at base 753 and that $nei_{wt2}PA\text{-}C_{1223}$ lacks five nucleotides on the 3'-end. Translation of SEQ ID NO:79 indicates that nucleic acid molecule $nei_{wt2}PA\text{-}C_{1223}$ also encodes $Pei_{wt1}PA\text{-}C_{388}$ (SEQ ID NO:77), assuming an open reading frame having a first codon which spans from nucleotide 3 through nucleotide 5 of SEQ ID NO:79 and a termination codon which spans from nucleotide 1167 through nucleotide 1169 of SEQ ID NO:79. Because SEQ ID NO:79 is only a partial gene sequence, it does not contain an initiation codon. The nucleic acid molecule having a coding strand comprising nucleotides 3 to 1166 of SEQ ID NO:79, denoted $nei_{wt2}PA\text{-}C_{1223}$, is identical to SEQ ID NO 78.

B. A nucleic acid molecule of 1233 nucleotides encoding a C-terminal portion of influenza PA-C cold-adapted equine influenza virus protein, denoted $nei_{ca1}PA\text{-}C_{1233}$, and having a coding strand having a sequence designated SEQ ID NO:80 was produced as described in as in Example 20, part A, except that the pCR®-Blunt cloning vector was used.

Translation of SEQ ID NO:80 indicates that nucleic acid molecule $nei_{ca1}PA\text{-}C_{1233}$ encodes a C-terminal portion of equine influenza PA protein of about 390 amino acids, referred to herein as $Pei_{ca1}PA\text{-}C_{390}$, having amino acid sequence SEQ ID NO:81, assuming an open reading frame having a first codon which spans from nucleotide 3 through nucleotide 5 of SEQ ID NO:80 and a termination codon which spans from nucleotide 1173 through nucleotide 1175 of SEQ ID NO:80. Because SEQ ID NO:80 is only a partial gene sequence, it does not contain an initiation codon. The region encoding $Pei_{ca1}PA\text{-}C_{390}$, designated $nei_{ca1}PA\text{-}C_{1170}$, and having a coding strand comprising nucleotides 3 to 1172 of SEQ ID NO:80, is represented by SEQ ID NO:82.

PCR amplification of a second nucleic acid molecule encoding a cold-adapted equine influenza PA-C protein in the same manner resulted in molecule $nei_{ca2}PA\text{-}C_{1233}$, containing one A to G base shift at base 953 as compared to $nei_{ca1}PA\text{-}C_{1233}$; this base shift does not result in an amino acid change so $Pei_{ca2}PA\text{-}C_{390}$, is identical to $Pei_{ca1}PA\text{-}C_{390}$ (SEQ ID NO: 81.)

C. Comparison of the nucleic acid sequences of the coding strands of $nei_{wt1}PA\text{-}C_{1228}$ (SEQ ID NO:76) and $nei_{ca1}PA\text{-}C_{1233}$ (SEQ ID NO:80) by DNA alignment reveals the following difference: an C to T base shift at base 753 of SEQ ID NO:80. Comparison of the amino acid sequences of proteins $Pei_{wt1}PA\text{-}C_{388}$ (SEQ ID NO:77) and $Pei_{ca1}PA\text{-}_{390}$ (SEQ ID NO:81) reveals the following difference: a W to R shift at amino acid 251 when relating to the C to T base shift at base 753 in the DNA sequences.

While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention, as set forth in the following claims.